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MESENCHYMAL STEM CELLS FOR THE TREATMENT OF MUSCULOSKELETAL PATHOLOGIES: FOCUS ON TENDINOPATHY

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ABSTRACT

Osteoarthritis and tendinopathy are common orthopaedic conditions with similar etiology, symptoms and clinical issues. They both are characterized by chronic inflammation and tissue degeneration, and the current conservative treatments do not effectively contrast the pathologic progression. The clinical need for innovative treatment has led to the development of regenerative medicine strategies, aimed to replacing or regenerating cells and tissues. In this setting, mesenchymal stem cells (MSCs) represents the most promising tool, thanks to their ability to proliferate and differentiate towards specific cell lineages, as well as their paracrine action on the immune system and on the tissue resident cells. Although the most used sources of MSCs are bone marrow and adipose tissue, they are present in almost all body compartments, with a perivascular localization. Indeed, MSCs have been recently described as pericytes, deputed to respond to damage and restore tissue homeostasis. The interest in new sources of MSCs resides in the discovery of their limited *in vivo* differentiation ability, especially towards specific lineages such as tenocytes and chondrocytes. To face this, subpopulations of tendon and cartilage derived cells were found to possess clonogenic and differentiation abilities, and they were proposed as possible candidates for specific tissue regeneration. Contrary to their well-known ability to

participate directly to tissue regeneration, the paracrine activity of these specific progenitors is still unclear, as well as their real nature as MSCs. Then, the first goal of this research was to identify the MSCs minimal criteria in these subpopulations, in terms of differentiation ability, immunophenotype and proliferation. We found that tendon stem/progenitor cells (TSPCs) possess these minimal criteria, and share the low immunogenicity and the immumodulatory ability with adipose derived stem cells (ASCs). On the contrary, while cartilage cells are the most effective in differentiate towards hyaline cartilage, they have low paracrine activity and thus a treatment based on the molecules released by ASCs could still result beneficial in case of joint inflammation or degeneration. The proof of concept for the application of ASCs conditioned medium, that is the complex of the molecules secreted during culture, was provided in an *in vitro* model of cartilage cell inflammation, resulting in a reduction of the catabolic response. After the characterization, we focused on the strategies to enhance the abilities of the progenitors with a therapeutic perspective. At first, we identified the role of different growth factors in the increase of TSPCs proportion within the tendon cell population during culture and in the induction of tenogenic differentiation. Then, we observed the *in vitro* effects of biophysical stimulations, such as extracorporeal shock waves (ESWs) and pulsed electromagnetic fields (PEMFs) on tendon cells, identifying a biological response with regenerative purport in terms of specific marker

expression and paracrine activity. On these bases, we investigated the possibility to use PEMFs to enhance the potential of resident stem/progenitors cells to contrast the pathology progression, in a specially developed *in vivo* model of tendinopathy. The preliminary results from this study provided promising data, supportive of further investigations. Finally, to fill the gap between the experimental setting and the clinical practice, uncultured ASCs, in the form of stromal vascular fraction, were tested in the treatment of Achilles tendinopathy in a clinical trial, providing results supportive of their efficacy. In conclusion, the present work allowed increasing the knowledge in the field of MSCs applied to the most common orthopaedic conditions, with a panoramic view ranging from the *in vivo* to the clinical aspects. This research will contribute in the development of new therapeutic opportunities for these high impact pathologies.

RIASSUNTO

L’artrosi e le tendinopatie sono patologie che presentano simili sintomi, quesiti clinici ed eziologia, entrambe caratterizzate da infiammazione e degenerazione tissutale. I trattamenti conservativi attualmente disponibili hanno dimostrato scarsa efficacia, portando interesse nello sviluppo di strategie di medicina rigenerativa, mirate a sostituire o rigenerare le cellule e i tessuti danneggiati. Le cellule staminali mesenchimali (MSC) rappresentano lo strumento più promettente per questi approcci, grazie alla loro capacità di proliferare e differenziarsi verso specifiche linee cellulari, oltre alle loro proprietà anti-infiammatorie e trofiche. Le principali fonti di MSC sono il midollo osseo e il tessuto adiposo, sebbene esse siano presenti in quasi tutti i distretti corporei, con localizzazione perivascolare. Infatti sono state recentemente descritte come periciti, cellule deputate a rispondere al danno tissutale ripristinando l'omeostasi. L'interesse verso fonti alternative di MSC è nato a causa della loro limitata capacità di differenziare *in vivo*, soprattutto verso la linea tenogenica e condrogenica. In questo contesto sono state identificate alcune sottopopolazioni di cellule tendinee e cartilaginee con abilità clonogeniche e differenziative, in grado di partecipare direttamente alla rigenerazione tissutale. Tuttavia, l'attività paracrina di questi progenitori, così come la loro reale natura di mesenchimali, è

ancora dibattuta. Il primo obiettivo di questa ricerca è stato quindi quello di identificare i criteri minimi tipici delle MSC in queste sottopopolazioni in termini di capacità differenziative, proliferative e di immunofenotipo. Le cellule staminali/progenitrici del tendine (TSPC) sono risultate in possesso di questi criteri, mostrando anche una scarsa immunogenicità e una capacità immumodulatoria tipica delle ASC. Al contrario, le cellule cartilaginee possiedono una maggior capacità di differenziazione verso la cartilagine ialina, ma hanno bassa attività paracrina rispetto alle ASC. Perciò, aggiungendo mezzo condizionato da ASC in un modello *in vitro* di infiammazione su condrociti, è stato possibile ridurre la concentrazione dei marcatori del catabolismo cartilagineo. Una volta caratterizzate, ci siamo concentrati sulle strategie per aumentare le potenzialità terapeutiche di queste cellule. Inizialmente abbiamo identificato il ruolo di diversi fattori di crescita nell'aumento della proporzione di TSPC all'interno della popolazione di cellule tendinee, ma anche nella induzione della differenziamento tenogenico. Successivamente, abbiamo osservato gli effetti *in vitro* delle stimolazioni biofisiche, come le onde d'urto extracorporee (ESWs) e i campi elettromagnetici pulsati (PEMF) sulle cellule tendinee, individuando l'induzione di una risposta biologica in senso rigenerativo. Su queste basi, abbiamo studiato la possibilità di utilizzare i PEMF per aumentare il potenziale delle TSPC residenti nel contrastare la progressione patologica in un modello *in vivo* di tendinopatia appositamente sviluppato. I risultati preliminari di questo

studio supportano l'efficacia di questo approccio. Per colmare il divario tra le condizioni sperimentali e la pratica clinica, le ASC, sotto forma di frazione stromale vascolare ovvero prima del passaggio in coltura, sono state applicate nel trattamento della tendinopatia d'Achille in uno studio clinico, ottenendo risultati a sostegno della loro efficacia. In conclusione, il presente lavoro ha contribuito ad aumentare le conoscenze nel campo delle MSC applicate alle patologie ortopediche più comuni, tenendo in considerazione tutte le problematiche presenti dagli scenari *in vitro* a quelli clinici. Partendo da questa ricerca sarà possibile in futuro sviluppare nuove strategie terapeutiche per queste patologie.

1.INTRODUCTION

1.1 Regenerative Medicine

According to the definition by Mason and Dunnill, regenerative medicine is the "process of replacing, engineering or regenerating human cells, tissues or organs to restore or establish normal function" [Mason C and Dunnell P, 2008]. The possibility to regenerate the body has always represented a recurrent dream in human history. Herodotus firstly described the "Fountain of Youth" to be located in ancient Aethiopia and serving the mythological tribe of Macrobians to live up to 120 years [Herodotus, Book III: 23]. As bequeathed by the popular culture, this desire to rejuvenate and be healed from the consumption of time and diseases led kings and adventurers to spend money and lives in the search of such a mighty power, with the greatest example in the most famous legendary expedition of Juan Ponce de Leòn, who explored the Caribbean Islands and Florida in the early XVI Century [Greenspan J, 2013]. Then, slowly but constantly, the myth drifted into science. The swiss physician Philippus Aureolus Paracelsus, contemporary to Ponce de Leòn, laid the foundations of regenerative medicine concept in his *Die Grosse*

Wundartznei, stating that "the heart heals the heart, lung heals the lung, spleen heals the spleen; like cures like" [Paracelsus PA, 1536]. Indeed, the idea that elements within the organs would heal the organs themselves appears prophetical, since the "cell theory" was completed just two centuries later in 1858 by Rudolf Virchow [Virchow R, 1858], and the "stem cell" was described for the first time in 1909 by Alexander Maximov [Maximov A, 1909] providing the first clue about the mechanism behind this possibility. In the same period, Cohnheim provided the first observation of bone marrow derived cells involvement in wound healing [Conheim J, 1867]. Then, in 1961, these cells were described as adult stem cells by McCulloch and Till [McCulloch EA and Till JE, 1960; Becker AJ et al., 1963], and they were used by Friedenstein to produce osteogenesis *in vitro* [Friedenstein et al., 1966]. Since then, research over this cell type gather a growing interest, until they were named "Mesenchymal Stem Cells" (MSCs) for the first time in 1991, because of their close relation to all tissues of mesenchymal origin, and contextually described them as capable of cartilage tissue production, depending on the environmental conditions [Caplan AI, 1991]. A plethora of studies originated from these early reports, and nowadays the evolution of knowledge about MSCs is advancing, opening new scenarios and fueling new doubts.

Today, regenerative medicine is referred to the field of application of all the tools able to contrast tissue degeneration, either of genetic, traumatic or physio-pathological origin. A number of approaches of biological,

biochemical and technological nature have been taken in account, such as platelet rich plasma (PRP), recombinant bioactive molecules, gene therapy and the most advanced biomaterials. Among the others, MSCs has proven to be a multimodal tool that, alone or in combination with these factors, provide the most promising results in pre-clinical and clinical evaluations, and represent the fundamental element of regenerative medicine.

1.1.1 What are MSCs?

The discussion about the real nature of MSCs is ongoing since their discovery in bone marrow in the XIX century. The acronym "MSCs" for Mesenchymal Stem Cells, coined in 1991, was recently proposed to be changed as well. Today some refers to MSCs as Mesenchymal Stromal Cells [Lindner U et al., 2010], while Prof. Caplan himself recently introduced the concept of MSCs as Medicinal Signaling Cells, to comply with the most recent findings in the field that will be describe later [Caplan AI, 2010]. These name changes follow the insights provided in the last decade about their therapeutic potential and, in particular, the discovery of their action as "drugstore", intended as the ability to release bioactive molecules in response of tissue damages [Caplan AI and Correa D, 2011]. Moreover, the discovery of MSCs population in almost all body compartments, such as muscle, adipose tissue, synovium, derma and tendon [Zengin E et al., 2006; Bi Y et al., 2007; Osyczka AM et al., 2002;

Zuk PA et al., 2001; De Bari C et al., 2001; Lu HH et al., 2003], brought the attention to the perivascular localization of MSCs, as confirmed by the direct correlation between the level of vascularization and the density of MSCs within each tissue [Crisan M et al., 2008; Traktuey DO et al., 2008]. Then, it recently became clear that "all MSCs are pericytes" [Caplan AI, 2008]. Nevertheless, the MSCs acronym, as well as the minimal criteria to define a MSCs population as given in the position paper issued by the International Society for Cellular Therapy in 2006, are still valid and consistent throughout literature. Those minimal criteria, that is the ability to adhere to plastic, the tri-lineage differentiation capacity (osteogenesis, chondrogenesis and adipogenesis) and the expression of a clear pattern of surface markers ($CD73^+$, $CD105^+$, $CD90^+$, $CD34^-$, $CD45^-$, $CD14^-$, $CD11b^-$, $CD79a^-$, $HLA\text{-}DR^-$) [Dominici M et al., 2006], are still used very frequently to characterize these cells. However, pretty forthwith the researchers realized that that immunophenotype was not sufficient to describe such a complex MSCs heterogeneity. For example, the expression of CD34, commonly associated with hematopoietic cells, was reported in subpopulations of MSCs [Boquest AC et al., 2006; Varma MJO et al., 2007; Yoshumura K and Shiguera T, 2006; Mitchell JB et al., 2006]. Accordingly, a study by Zimmerlin and colleagues identified two different subset of adipose derived mesenchymal stem cells (ASCs), based on the alternative expression of this marker and CD146, where $CD34^+/CD146^-$ cells are stromal super adventitial cells, while $CD34^-/CD146^+$ cells are the pericytes

[Zimmerlin L et al., 2013]. The same was observed in derma-derived MSCs [Al-Nbaheen M et al., 2013]. Moreover, freshly isolated cells from bone marrow aspirate and stromal vascular fraction of adipose tissue, appear to be enriched in fibroblasts colony forming units when sorted by the endothelial antigen Stro-1 [Simmons P, 1991; Gronthos S et al., 1994; Gronthos S et al., 2001; Lin CS et al., 2010]. A complete and plausible *in vivo* phenotype would then include CD146, CD90, CD105, CD73, CD44 and Stro-1 expression and the lack of CD45, CD115 and CD14, CD11b, CD79a and HLA-DR. The doubt on CD34 expression still persists and it is probably correlated to the presence of slightly different subpopulations in the complex MSCs pool [Murphy MB et al., 2013]. Indeed, some of these cells appear to be committed toward a particular lineage and can be considered progenitors [Deschaseaux F et al., 2010, Izadpanah R et al., 2006, Cao B et al., 2003], while others, i.e. the pericytes, are sensing cells able to initiate a paracrine response when exposed to signals of tissue damage [Caplan AI, 2016].

The role of stem cells in adult organisms is tissue homeostasis. This role comprise a complex set of actions and properties, starting from the ability to generate a progeny of differentiated cells deputed to substitute the ones naturally expiring. In physiological conditions, this mechanism allows endogenous tissue rejuvenation and modification following age or functional changes. Similarly, in pathological conditions, tissue homeostatic activity comprise the cascade of events that allow these cell

to sense the presence of damages and respond with proper biological counteractions [Caplan AI and Dennis JE, 2006]. In particular, MSCs are able to sense the presence of danger-associated molecular patterns (DAMPs) through specific receptors for cytokines and chemokines that allow their recruitment to the site of injury too [Hengartner NE et al., 2015]. Once activated, MSCs could exert immunomodulatory action, representing the main defense against the auto-immune damages, as well as provide trophic stimuli for recruit and enhance the growth of tissue specific progenitors, favoring the establishment of a regenerative microenvironment [Caplan AI, 2016].

1.1.2 Therapeutic Potentials of MSCs

The possible application of homeostasis-inducing agents would be useful in the treatment of a wide variety of pathologies characterized by tissue loss or degeneration. Nevertheless, tissue homeostasis is a complex tasks that require further capability beyond proliferation and differentiation, and the most recent discoveries in MSCs fields have underlined their indirect participation to the regenerative process through paracrine action on resident cells [Murphy MB et al., 2013; Caplan AI and Correa D, 2011].

On the other side, the MSCs ability to directly differentiate into tissue-specific cells has to be also considered while proposing a MSCs-based therapy [de Girolamo L et al., 2016; Munir H and McGettrick HM, 2015;

Peired AJ et al., 2016; Tang QO et al., 2012], even because it would be impossible to separate the direct and indirect MSCs action in the context of a particular therapeutic application. For example, when using MSCs to regenerate bone, the ability to differentiate towards the osteogenic lineage also benefits from the MSCs capacity in the induction of angiogenesis, representing a critical aspect in the bone formation process [Avolio E et al., 2016]. Then, in the following paragraph the description of MSCs potential will be provided as based on the intended use, to put order between the different therapeutic purposes, but it must be considered that any clear separation would result questionable under profound scientific investigation.

Tissue Engineering

Tissue engineering is the branch of regenerative medicine that has remained faithful to the original intent of using stem/progenitor cells to artificially rebuild damaged biological tissue. It was defined in early 1990s as an interdisciplinary field that combine the principles of life sciences and engineering in order to develop biological substitutes to restore, maintain and improve tissue function [Langer R and Vacanti JP, 1993]. The ability of MSCs to proliferate and differentiate towards a wide variety of lineages indeed proved the feasibility of this approach, in both *in vitro* and *in vivo* experiments. Bone tissue was the first to be investigated [Friedenstein et al., 1966], and gather today a broad consensus in terms of success ratio.

Indeed, MSCs derived from either bone marrow or adipose tissue, alone or in combination with scaffolds, were successfully applied to the treatment of calvarial defect in different animal models [Chen KY et al., 2013; Dumas A et al., 2009; Zhang H et al., 2016; Cowan CM et al., 2004; Yoon E et al., 2007; Choi JW et al., 2014; Levi B et al., 2010]. Satisfying results were also obtained in the treatment of long bone defect [Fernandes MB et al., 2014; Wang C et al., 2010; Choi HJ et al., 2011; Cruz AC et al., 2015; Schubert T et al., 2013], despite the higher complexity related to the weight bearing conditions, and the few clinical reports available confirm the efficacy of MSCs in bone regeneration [Giannotti S et al., 2013; Gallego L et al., 2012; Lendeckel S et al., 2004; Taylor JA, 2010].

Conversely, the effectiveness MSCs-based tissue engineering of cartilage is still debated. BMSCs have been using in clinical practice for the treatment of chondral defects for many years [Steadman JR et al., 2003; Yang HS et al., 2011], and when properly cultured *in vitro*, BMSCs and ASCs express the typical markers of hyaline cartilage such as collagen type II, aggrecan and proteoglycans [Johnstone B et al., 1998; Johnstone B and Yoo JU, 1999; Pittenger MF et al., 1999; Barry F et al., 2001]. Nevertheless, the proportions of these extracellular matrix component are extremely different from those of native hyaline cartilage [Kafienah W et al., 2008; Kafienah W et al., 2002; Kafienah W et al., 2003; Marijnissen WJ et al., 2002; Riesle J et al., 1998], and the *in vitro* differentiation protocols

induces also the expression of hypertrophy markers such as collagen type X, alkaline phosphatase and metalloproteinases [Johnstone B et al., 1998; Mackay AM et al., 1998; Mwale F et al., 2006 Aug; Mwale F et al., 2006 Sep; Yoo JU et al., 1998; Pelltari K et al., 2006; Mueller MB and Tuan RS, 2008]. Moreover, mineralization, vascularization and ossicles formation were observed in SCID mice after MSCs-engineered cartilage transplantation [Pelltari K et al., 2006]. Taken together these evidences support the idea that cartilage differentiation of MSCs resemble the early phases of endochondral ossification rather than hyaline cartilage repair, through the development of fibrocartilage intermediate tissue [Somoza RA et al., 2014].

Comparing to bone and cartilage, tendon tissue engineering is under-investigated, although tendon surgeons claim for innovative techniques to ameliorate the rate of treatment success. Culture of MSCs in presence of BMP-12 and BMP-14 resulted in the increased expression of tendon markers and morphological changes to tendon-like cells [Tan SL et al., 2012; Violini et al., 2009]. *In vivo* reports sustain so far the effectiveness of ASCs and BMSCs application to tendon regeneration [Lacitignola L et la., 2014], but many aspects are still unclear and more basic science is needed to move the production of bio-fabricated tendons forward.

From a general and traditional point of view, tissue engineering relies on a triad of fundamental elements: cells, biochemical stimuli and biomaterials. Then, MSCs are just a single part of this equation and they

could be easily substituted when more effective biological agents will be identified. For example, induced pluripotent stem cells (iPS) have been proposed, for their enormous proliferative and differentiation potential, which is similar to that of embryonic stem cells [Fishman JM et al., 2013; Martins AM et al., 2014]. Nevertheless, while the use of iPS still arise safety and regulatory issues, the possibility to apply tissue specific progenitors is a much more feasible technique. Mature cells have been proposed as tools for regenerative medicine. In fact, the articular chondrocytes implantation (ACI or MACI if matrix-assisted), was first invented in the 1990s and so far satisfactory clinical outcomes were reported, even in presence of somehow controversial results among groups [Kon E et al., 2012]. These controversies could be partially solved by the use of lately discovered tissue specific cartilage progenitors instead of mature articular chondrocytes, since they would retain a greater proliferative and clonogenic potential [Jiang Y and Tuan RS, 2015]. Then, there is a growing interest in the identification and the selection of tissue specific progenitors, since they represent the most promising tool for future application of tissue engineering approaches.

Cell Therapies: cell as a drug

As said, the discovery of MSCs paracrine action has broadened their therapeutic potential, beyond the one taken in account by the more traditional tissue engineering approach. Indeed, while the most recent

reports has demonstrated the limitation of proper MSCs application to cartilage regeneration, the possibility to restore the endogenous tissue homeostasis by the release of bio-active molecules represents a new field of opportunities.

The first report of bioactive molecules released by MSCs was provided by Heynesworth and colleagues in 1996, and regarded BMSCs treated with dexamethasone and IL-1α, that were able to produce cytokines (IL-6, IL-11) and growth factors (G-CSF, M-CSF, SFF, LIF) [Heynesworth et al., 1996]. These factors, released by MSCs resulted important in supporting hematopoiesis [Majumdar MK et al., 1998], and since then the ability of these cells to release a great variety of bioactive molecules with many different effects has been investigated and described [Murphy MB et al., 2013]. Indeed, MSCs could elicit trophic, antiapoptotic, immunomodulatory, pro-angiogenic and anti-microbial effects. The trophic ability comprises the release of mitogen proteins such as transforming growth factor β (TGFβ), epithelial growth factor (EGF), hepatocyte growth factor (HGF), insulin like growth factor 1 (IGF-1) and basic fibroblast growth factor (bFGF), that are able to induce proliferation of endothelial, epithelial and fibroblast cells [Doorn J et al., 2011; Caplan AI and Bruder SP, 2001; Haynesworth SE et al., 1996; Holgate ST et al., 2000]. The release of vascular endothelial growth factor (VEGF) and angiopoietin-1, in addition to those listed before, indicates the ability of these cells in sustaining angiogenesis by recruitment of vascular

endothelial cells [Chen L et al., 2008]. The production of other proteins such as keratinocyte growth factor, stromal cell-derived factor 1 (SDF-1) and macrophage inflammatory protein 1, suggested that MSCs could play a role not only in tissue regeneration, but also in promoting the remodeling of initially formed scar tissue the healing process. Indeed, while the regeneration can spontaneously occur *in vivo*, the quality of tissue depends on number and activity of stem cells at the injury site, which dramatically decrease with aging [Caplan AI, 2016]. The MSCs ability to sustain tissue resident cells does not only involve the proliferative aspect, but also apoptosis inhibition, in cases of hypoxia-, chemical-, mechanical- and radiation-induced cell death. The molecules supposed to intervene in these cases are IGF-1 and IL-6, that enhance the production of protein kinase B (Akt) and nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) [Mitsiades CS et al., 2002; Gnecchi M et al., 2006], triggering Wnt signaling with an anti-apoptotic significance [Mirotsou M et al., 2007; Alfaro MP et al.,2008; Zhang Z et al., 2009;]. Moreover, the production of VEGF, HGF and TGFβ1 is crucial to rescue endothelial cells from apoptosis in hypoxic conditions [Rehman J et al., 2004].

For what concern the immunomodulatory properties of MSCs, beyond their ability in avoiding targeting from host immune system when used in an allogeneic (or xenogeneic) environment [Aggarwal S and Pittinger MF, 2005; Koc ON et al., 2002; Le Blanc K et al., 2008; Ringden O et al., 2006]

thanks to the low expression of HLA class I and II molecules [Dominici M et al., 2006; Siegel G et al., 2009], they can release a number of cytokines that could greatly vary depending on microenvironment. In fact, in response to pro-inflammatory cytokines such as interleukine-1 (IL-1), IL-2, IL-12, tumor necrosis factor α (TNFα), and interferon γ (IFNγ), MSCs can produce different anti-inflammatory proteins and growth factors targeting almost all immune system cells [Aggarwal S and Pittenger MF, 2005; Iyer S and Rojas M, 2008; Uccelli A et al.,2008; Weiss DJ et al.,2011; Yagi H et al., 2010; Guan XJ et al., 2013]. These molecules act on immune cells by diminishing the production of damage-associated proteins and favoring the release of anti-inflammatory mediators, such as prostaglandin 2 (PGE2), nitrous oxide (NO), indoleamine 2,3-dioxygenase (IDO), IL-4, IL-6, IL-10, IL-1 receptor antagonist (IL-1RA) and soluble tumor necrosis factor α receptor (TNFR). Taken together all these molecules are able to induce the maturation of a higher number of regulatory T cells (Treg) with respect to inflammatory cells such as lymphocyte T ($CD4^+/CD8^+$), B cells, natural killer (NK) cells, dendritic cells, monocytes and macrophages [Iyer S et al., 2008; Spaggiari GM et al., 2009; Selmani Z et al., 2008]. Moreover, they could enhance the transition of T helper cells type 1 to type 2 modifying the T_H1/T_H2 balance. This results in a great improvement of tissue regeneration of cartilage and soft tissues, and in the reduction of immune disease symptoms [Tollervey JR et al., 2011; Tidball JG et al., 2010; Choi EW et al., 2011; Kong Q et al, 2009]. Similarly,

the reduction of IFNγ and the increase of IL-4 could drive the shift from pro-inflammatory macrophages maturation (M1) to anti-inflammatory (M2) phenotype, thus favoring healing and tissue regeneration [Mantovani A et al., 2004; Tidball JG et al., 2010; Mokarram N et al,2012; Wang Y et al, 2007; Sica A et al., 2008; Chaudhuri B et al., 2012;].

The most recent discover about immunological feature of MSCs is the supposed anti-microbial action. Indeed, MSCs produce the peptide LL-37, belonging to the cathelicidin family, that is commonly expressed by epithelial cells and phagocytic macrophages to contrast Gram+ and Gram- bacteria [Frohm Nilsson M et al., 1999; Nijnik A et al., 2009; Coffelt SB et al., 2009]. The production of indoleamine 2,3-dioxygenase (IDO) is another possible pathway of the anti-microbial activity of MSCs [Meisel R et al., 2011].

Taken together, these data support the application of MSCs to a wide number of pathologies, involving inflammation, tissue damage, ischemia, degenerative diseases, and sepsis. It must be considered that MSCs derived from different sources could present peculiar patterns or levels of paracrine agents, and that the *in vitro* culture condition can modify the panel of the released molecules. The choice among cell sources and application of specific culture protocols would then allow for a tailoring of MSCs secreted effectors, permitting the identification of specific therapeutic procedures for each different therapeutic purpose [Konala 2016].

The transition from the traditional tissue engineering to the cell therapy view represents not only a conceptual change, but also moved MSCs therapy closer to application, as confirmed by the percentage of open clinical trials on clinicaltrials.gov. Indeed, a total of 178 open studies can be found when searching for "MSCs injection", while just 10 are listed for "MSCs scaffold" [clinicaltrials.gov, retrieved in January 2017]. A number of factors, including technological, economical and regulatory, favors the clinical feasibility of this approach.

MSCs as therapeutic target

From a regulatory point of view, the possible application of most of the proposed tissue engineering and cell therapy solutions is still uncertain. Clinical applications involving extensively manipulated cells or non-autologous or non-homofunctional cell transplantation should follow the strict regulation of the Advanced Therapy Medicinal Products (ATMPs) as defined by the European Directive 2001/83/EC and the amending Directive 2009/120/Ec, together with the Regulation (EC) number 1394/2007. Then, the possibility to enhance the potential of endogenous mesenchymal and progenitor cells, which physiologically reside in tissues, represents an opportunity to avoid treatment invasiveness and regulatory issues. Since MSCs are present in almost all body compartments, any damaged tissue would benefit from an increase of their intrinsic reparative activity. A growing interest is rising on this concept, and many

tools, already used in clinical practice, have been identified as possible effectors for the stimulation of endogenous MSCs. In particular, for what regard the orthopaedic field, biophysical stimulation represents a well-established approach for bone non-union fractures and necrosis [Zelle BA, et al 2010; Hannemann PF, 2014]. *In vitro* findings showed that biophysical stimulation was able to enhance proliferation, differentiation and paracrine activity of MSCs from different sources, thus supporting the idea that tailoring these approaches on MSCs response would result in an enhanced tissue homeostasis restoration [Viganò M et al., 2016]. These treatments comprise Extracorporeal Shock Waves (ESWs) and Pulsed ElectroMagnetic Fields (PEMFs) that have proven efficacy in many orthopaedic pathologies such as bone non-union, OA and tendinopathy. Despite their mechanism of action is still unclear, they resulted capable to reduce the production of inflammatory mediators and increase trophic and immunomodulatory action of MSCs, as well as proliferation of cells derived from cartilage, bone and tendon tissues [Viganò et al., 2016]. In addition, biological treatments for MSCs stimulation have been also proposed, with promising results. For example, platelet rich plasma (PRP) is often applied as an adjuvant in cell therapy, and its capability to provide growth factor supplementation can support MSCs proliferation and delay senescence *in vitro*, thus indicating its potential in this approach [Rubio-Azpeitia E and Andia I, 2014].

1.2 Musculoskeletal Pathologies

The definition of musculoskeletal diseases includes a number of pathological conditions affecting bone, cartilage, muscle, tendon and ligaments. These conditions range from trauma to complex systemic diseases such as rheumatoid arthritis, involving many different biological pathways. While satisfactory treatments for most of these conditions are available today, certain pathologies involving tissue degeneration, such as osteoarthritis and tendinopathy are far from being properly solved, expecially in young patients where anatomy and function has to be preserved. Alternatively to surgical solutions, which are often effective but invasive, the conservative treatment fails in counteracting tissue degeneration, allowing for temporary pain relief only [Gallagher B et al., 2015; van Walsem A et al., 2015]. In this context the ability of MSCs to promote endogenous tissue regeneration, contrast inflammation, and, in some cases, participate directly to replace tissue-specific cells appear an optimal therapeutic solution.

Osteoarthritis and tendinopathy are extremely common orthopaedic conditions that shares many features in terms of etiology, symptoms and clinical issues. Indeed, they both are characterized by chronic inflammation and degeneration in a context of poorly cellularized and vascularized tissue such as cartilage and tendon.

1.2.1 Osteoarthritis

Osteoarthritis (OA) is a whole joint pathology, involving cartilage, synovium and in late stages the subchondral bone. It affects over 250 million people in the world and it is growing, with a particularly high incidence among women over 55 years of age, representing a social and economic burden [Hunter DJ et al., 2014; Vos T et al., 2012, 2015, Srikanth VK et al.,2005; Lohmander LS, 2013]. Its etiology could be ascribed to many different factors, including aging, body mass index (BMI), gender, mechanical loading, excessive physical activity, and nutrition [Yucesoy B et al., 2015]. The variable nature of these factors lead to the classification of the disease into primary and secondary OA. Primary OA includes all cases deriving from genetic inclination and biochemical profile, while secondary OA originate from trauma (post-traumatic OA, PTOA), chronic inflammation, or metabolic disorders [Kapoor M et al., 2011]. Primary and secondary OA share symptoms such as pain, inflammation, joint stiffness and loss of function. Moreover, at later stages, joint space narrowing, synovial thickening, and the generation osteophytes can be observed, as well as damage to subchondral bone, and limitation to work and life activities [Crema MD et al., 2011] appear more and more evident. The two main components of the pathological progression are joint inflammation and cartilage degeneration. During OA onset, the articular chondrocytes , that are usually quiescent and are responsible for cartilage extracellular matrix (ECM) production, start to proliferate and increase

the synthesis of proteins with a catabolic action, such as Matrix Metalloproteinases (MMPs), reducing at the same time the production of ECM [Goldring MB and Marcu KB, 2009; Mort JS et al., 2016; Troeberg L and Nagase H, 2012]. Moreover, the metabolic re-activation of chondrocytes leads to an imbalanced homeostasis, with cellular hypertrophy, which can induce articular cartilage calcification [Dreier R, 2010]. Taken together, these events imply the degradation of cartilage tissue, causing the formation of debris in the synovia, eventually triggering inflammation [Rosenthal AK, 2011]. The inflammatory process involved in OA comprises the migration of immune cells into the synovia and the production of inflammatory agents, mainly IL-1β and TNFα [Bondeson J et al., 2006; Ishii H et al., 2002]. These molecules induce the production of secondary inflammatory mediators such as cyclooxygenases (COX1 and COX2), prostaglandins (PGE2) and reactive oxygen species (ROS) that could stimulate apoptosis of chondrocytes [Reed NK et al., 2014; Sokolove J and Lepus CM, 2013; Wang P et al., 2013; Intekhab-Alam NY et al., 2013]. In response to these events, chondrocytes produce TGFβ and BMP-2, as well as VEGF. Despite the regenerative capability of these molecules, in this context they could induce the formation of fibrotic tissue or ossification [Ripamonti 2016, Durani 2008]. Indeed, the increased bone density and the presence of osteophytes are typical markers of late stage OA [Burr DB and Gallant MA, 2012; Castañeda S et al., 2012].

Current alternatives in OA treatment comprise conservative and surgical approaches. The application of non-steroidal anti-inflammatory drugs (NSAID) is the most popular conservative treatment, but they are not effective in the reduction of tissue degeneration, and in addition, they cause a number of side effects that made it unsustainable for long periods of treatment [Lanas A et al., 2011; Mandegaran R et al., 2013]. Another pharmacological option is viscosupplementation, usually by intra-articular injection of hyaluronic acid (HA). HA treatment aims to restore the protective function of synovial fluid and improve joint functionality. Unfortunately, this approach proved to have just minimal and short beneficial effects on OA symptoms [Rutjes AWS et al., 2012; Bannuru RR et al., 2014]. Many different surgical solutions have been proposed. Joint arthroplasty is known to be the definitive treatment for OA, but due to its irreversible nature, it should be applied just in late stage OA and in older patients [Rönn K et al., 2011]. Less invasive attempts to cease or at least delay the degeneration of early OA were proposed, starting from arthroscopic debridement, although it was indicated as ineffective [Moseley JB et al., 2002]. Later on, cell-based approaches, such as microfractures of the subchondral bone to recruit MSCs from bone marrow to the damaged joint to stimulate tissue repair [Steadman JR et al., 1999], were introduced. Tissue engineering approaches, such as ACI and MACI, to regenerate articular cartilage [Ossendorf C et al., 2011] were also studied and used for some years. However, despite their

effectiveness, these techniques are invasive and present issues related to donor side morbidity [Ondresik M et al., 2016] and thus currently they are very rarely used.

In this context, the possibility to develop an effective conservative treatment to contrast tissue degeneration in early OA, opened the research field about MSCs potential.

1.2.2 Tendinopathy

Tendon disorders affect from 16% to 21% of the worldwide populations [Urwin M et al., 1998; Chard MD et al., 1991], and represent the 30%-50% of total sports injuries [Kannus P, 1997a]. Achilles, rotator cuff and patellar tendons are the most frequently affected by degeneration, but also tibialis posterior and fingers flexor tendons are subject to laceration at all ages [Docheva D et al., 2015]. The classification of tendon diseases is still debated; in the past they were usually divided in “tendinitis” in case of obvious inflammation and “tendinosis” when degeneration was more evident [Maffulli N et al., 1998]. Today, it is known that these two components are concurrent, and then the term “tendinopathy” allows for a better description of the whole condition [Rees JD et al., 2014; Riley G, 2004]. In fact, infiltration of immune cells, like macrophages and lymphocytes, mainly in the bursa or paratenon, was observed in chronic tendinopathy, as well as the presence of inflammatory mediators such as Substance P, MMPs and COX [Rees et al., 2014].

One of the problems with tendon disease description is the difficulty to perform biopsies during the pathological progression. According to the most recent findings, degeneration begin because of endogenous (BMI, abnormal weight bearing pattern or joint laxity) or exogenous factors (trauma and overuse) [Kannus P, 2007b; Kannus P, 2007c; Harring SA et al., 1987; Selvanetti A et al., 1997; Kjaer M, 2001]. All these factors could act either on matrix, causing micro- or macro-trauma at the fibers level, or on tendon resident cells, lowering their tissue homeostatic ability. In combination, these events lead to primary tissue damage that cannot be efficiently counteracted by resident cells that produce a reparative response leading to a low-quality/scar tissue, for example through an excessive production of TGFβ1 [Riley G, 2004; Caplan AI, 2016; Evans CH, 1999; Durani P et al., 2008].

After a trauma, the first occurring events are hematoma formation and infiltration of immune cells and fibroblasts from tendon surroundings [Lin TW et al., 2004]. These cells are recruited by the secretion of cytokines such as IL-1β and IL-6, as well as neovascularization is initiated by the production of angiogenic factors (VEGF), leading to the establishment of newly formed fibrous tissue at the injury site [Evans CH, 1999]. The role of angiogenesis in tendon regeneration is disputed, but there is a consensus about its importance in the first phases of tendon healing [Fenwick SA et al., 2002]. Indeed, a reduced vascularization was associated with ruptures of supraspinatus and Achilles tendons [Kannus

P, 1997b; Brooks CH et al., 1992; Ahmed IM et al., 1998; Leadbetter WB, 1992; Fenwick SA et al., 2002], whereas increased vascularization was described in chronic tendon disorders [Astrom and Rausing, 1995; Movin T et al., 1997]. During the early phase of the reparative response, when the fibrous tissue generation occurs, an increased cellularity and the production of collagen type III and proteoglycans are the first responses aimed to restore the ECM. These events are induced by growth factors such as BMPs, FGF, TGFβ and IGF-I [Evans CH, 1999]. The substitution of the fibrous tissue with proper tendon tissue, in particular through the substitution of collagen type III with collagen type I represents the last stage of tendon healing and definitive proper tissue maturation [Docheva D et al., 2015]. Any obstacle to this process, such as repetitive trauma or disease severity and patient own characteristics [Lin TW et al., 2004; Voleti PB et al., 2012; Yang G et al., 2013] could divert from tissue regeneration to the establishment of a chronic disease or tendon rupture. Treatment options for tendinopathy comprise a series of approaches reflecting the variability of symptoms and pathological stages. Indeed, he possibility to use a conservative treatment able to restore tissue homeostasis and prevent the pathological degeneration would be of great impact on the social and economic burden represented by tendon disorders, because of their frequency in the population and the difficulties in apply an effective treatment [Docheva 2015].

Eccentric exercise programs have been shown to improve pain and functionality in several studies; more recently, the coupling of eccentric and concentric exercise has shown an ameliorative outcome with respect to eccentric alone [Malliaras P et al., 2013]. Nevertheless, many patients remain unresponsive and the evidences are still somewhat conflicting, mainly due to protocol differences and the unknown mechanism of action [Malliaras P et al., 2013]. Moreover, this particular approach require a high patient's compliance, since it requires great will to respect a demanding training program and thus it is not applicable to all patients [Rees JD et al., 2006]. Among the pharmacological approaches, NSAIDs are a common symptomatic strategy to treat pain in tendinopathy, but their efficacy in both functional restoration and pain relief is controversial [Wang JC and Shapiro MS, 1997; Vogel HG, 1977; Carlstedt CA et al., 1987; Forslund C et al., 2003; Kulick MI et al., 1986]. Similarly, corticosteroid administration showed efficacy in the initial phases in different clinical trials, but no differences emerged with respect to placebo at longer time points [Buchbinder R et al., 2003; Assendelft WJ et al., 1996; Hay EM et al., 1999; Stahl S and Kaufman T, 1997]. Considering the great incidence of side effects related to the use of corticosteroid [Canoso JJ, 2003], this approach is not recommended as definitive treatment for tendon disorders [Speed CA, 2001]. Other conservative treatments comprise the use of biological preparations such as PRP or autologous blood. However, despite the number of studies showing satisfactory results and significant

improvement, these approaches failed in demonstrating any evidence for their use in the treatment of tendon pathologies [Moraes VY et al., 2014; Gholami M et al., 2016]. Further conservative approaches comprise cryotherapy and biophysical stimulations. While cryotherapy appear indicated to reduce pain and swelling immediately after injury, it has no clear beneficial effect in the short and long term [Rees JD et al., 2006]. On the contrary, extracorporeal shock waves (ESWs) were reported to be as effective as surgical approaches in the long term and also being equal (or superior) to other conservative approaches such as corticosteroid injection and training, in absence of the drawbacks given by these protocols [Mani-Babu S et al., 2015; Gerdesmeyer L et al., 2015]. Despite the *in vivo* mechanisms behind this success are still unclear, ESWs have shown *in vitro* the ability to induce tendon cell proliferation and production of anti-inflammatory cytokines that could explain these observations [de Girolamo L et al., 2014].

The surgical approach is mandatory in case of tendon ruptures, and even if most of the procedure are successful, the risk of re-occurrence is high [Marcheggiani Muccioli 2013]. Moreover, surgical treatments are suggested when patients is non-responder to available conservative approaches, and they comprise arthroscopic or more invasive techniques [Maffulli N et al., 2015].

1.2.3 Rationale of MSCs-based approaches for OA and Tendinopathy

The rationale to apply MSCs to the treatment of early OA and tendinopathy resides in their ability to contrast the two main components of these pathologies, inflammation and degeneration. In these cases, the unbalance in the physiological tissue homeostasis led to catabolic events with pathological significance. First, MSCs contrast the inflammation by different pathways, modifying the profile and behavior of immune cells and releasing anti-inflammatory molecules. Then, they are able to sustain the regeneration of damaged tissue by direct proliferation and differentiation or supporting those of resident cells, by paracrine action. Remarkably, the tissue regeneration induced by MSCs is characterized by scar-free tissue formation and would allow the healing process to generate a new tissue with comparable physiological and mechanical properties to the native one [Caplan 2015]. Moreover, in the search for non-invasive and conservative treatment, the possibility to target in situ endogenous MSCs, or tissue specific progenitors, with biophysical or biochemical stimuli recently emerged. This would represent another possible strategy to obtain enhancement of the regenerative capability of tissues and organs [Viganò M et al., 2016].

2. AIM OF THE RESEARCH

The main aim of this PhD research is the improvement of knowledge about the potential of Mesenchymal Stem Cells (MSCs) in the treatment of inflammatory and degenerative musculoskeletal pathologies. In particular, this research has attempted to exhaustively assess the feasibility of the paradigm “from bench to bedside”, taking in account all the passages required to apply MSCs at clinical level. This evaluation has included the possible differences among MSCs derived from different tissues, the different levels of manipulation (from “none” to “extensive”), and comprised the investigation over the most appropriate culture conditions, as well as the safety and efficacy in pre-clinical and clinical models.

Thus, the final purpose of this research has been to collect data and evidences to further support the clinical use of MSCs for the treatment of musculoskeletal pathologies that can benefit from this kind of approach.

The first step of the research has focused on a deep *in vitro* characterization of MSCs and/or tissue specific progenitors from different sources, such as adipose tissue, tendon and cartilage, focusing on their differentiation and paracrine abilities, in order to assess their potential in different regenerative medicine applications (*Aim 1*).

Then, the possibility to enhance the properties of MSCs/progenitor cells through both biochemical and biophysical strategy has been investigated in order to increase the feasibility of tissue engineering and cell therapy approaches (*Aim 2*).

Then, pre-clinical and clinical studies have been performed to test the concrete efficacy of these approaches. A rat model of tendinopathy was established and used to assess the potential role of biophysical stimulation to promote tendon healing (*Aim 3*). Moreover, the safety and efficacy of a one-step cell therapy approach was tested in a clinical study, using the stromal vascular fraction of adipose tissue to treat Achilles tendinopathy (*Aim 4*).

3. Characterization of MSCs and progenitors from different sources (*Aim1*)

The first step to improve MSCs-based regenerative medicine approaches is the identification of the most suitable MSCs type for any given applications. Among the different MSCs source, bone marrow and adipose tissue, due to their large availability and low donor site morbidity, together with their efficient differentiation, proliferation and paracrine abilities represent the best sources. Nevertheless, questions arose about the suitability of these cells for cartilage tissue engineering, due to their propensity to form hypertrophic cartilage instead of hyaline, demonstrating the need for cells performing better in terms of specific differentiation. In this context, the discovery of tissue specific progenitors with stemness features in both tendon and cartilage opened the field to further development of regenerative strategies. In a first study a comparison between Tendon Stem/Progenitor Cells (TSPCs) and Adipose-derived Stem Cells (ASCs) was carried on to evaluate the differentiation and clonogenic properties of TSPCs and verify their nature as proper Mesenchymal Stem Cells (Manuscript 1, Stanco D et al., 2015 published on RegenMed). Moreover, further experiments were performed to assess

Aim 1

TSPCs immunogenicity and immunomodulatory properties with the aim to identify possible contraindications in their allogeneic use and to increase the understanding about their therapeutic abilities (Manuscript 2: Viganò et al., preliminary results of ongoing study). Similarly, we performed a comparison of clonogenic and differentiation abilities of articular cartilage progenitor cells with those of ASCs and Bone Marrow-derived Stem Cells (BMSCs). This study comprised the evaluation of cells derived from the intervertebral discs, in particular for nucleus pulposus (NP), cartilaginous end plate (CEP) and annulus fibrosus (AF). The reaction of all these cell types to inflammatory conditions was used as an indicator of cellular responses to pathological conditions. Lastly, the proof of concept for the possible use of ASCs conditioned-medium to treat cartilage diseases was provided, based on an *in vitro* inflammatory model (Manuscript 3: de Luca P et al., in preparation). The use of MSCs conditioned medium, containing all paracrine effectors of MSCs, would allow the exploitation of the trophic and immunomodulatory potential of these cells avoiding the throwbacks of direct cell transplantation, comprising the possible uncontrolled reactions of MSCs in a non-physiological environment, which are nowadays the main concern addressed by regulatory restrictions.

3.1 Manuscript 1: Multidifferentiation potential of human mesenchymal stem cells from adipose tissue and hamstring tendons for musculoskeletal cell-based therapy

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Aim: Adipose-derived stem cells (ASCs) have been deeply characterized for their usefulness in musculoskeletal tissue regeneration; recently, other mesenchymal stem cell (MSC) sources have also been proposed. This study compares for the first time human tendon stem/progenitor cells isolated from hamstring tendons with human ASCs.

Materials and Methods: Human TSPCs and ASCs were isolated from hamstring tendon portions and adipose tissue of healthy donors undergoing ACL reconstruction or liposuction, respectively (n=7). Clonogenic ability, immunophenotype and multidifferentiation potential were assessed and compared.

Results: Both populations showed similar proliferation and clonogenic ability and expressed embryonic stem cell genes and MSC surface markers. Tendon

stem/progenitor cells showed lower adipogenic and osteogenic ability, but after the chondrogenic differentiation, they produced more abundant glycosaminoglycans and expressed higher levels of aggrecan with regards to ASCs. The tenogenic induction with BMP-12 upregulated SCX and DCN gene expression in both populations. **Conclusion:** Our results demonstrate that waste hamstring tendon fragments could represent a convenient MSC source for musculoskeletal regenerative medicine.

INTRODUCTION

Although bone marrow is still the most common source of MSCs, in the last years researchers, driven by a constant quest for the "most convenient" source, have demonstrated that these cells can be identified in several alternative sites. In particular MSCs have been found in tissues that are discarded after surgical interventions, including adipose tissue, periodontal ligaments and deciduous teeth. Many studies have been performed to compare the features of MSCs from different origins, with the final aim to identify the possible best MSC tissue source for a given clinical situation. The choice of the most appropriate cell type is crucial, since, although mesenchymal stem cells deriving from different tissues share common properties, it is known that they are influenced by the surrounding microenvironment and tissue specific characteristics that can ultimately may influence the final treatment outcome. Moreover, age, site, gender and pathological conditions have been shown to affect the number, the proliferation potential and differentiation capacity of MSCs [1-4]. Recently, it has been shown that also discarded tendon fragments derived from knee ligament reconstruction [5] or tendon cuff repair [6] contains a MSC population, named

tendon stem/progenitor cells (TSCPs) [7]; this observation has allowed remarkable advancements in understanding the physiopathology of this tissue, as well as the possibility to use this cell source as a potential tool for tendon regenerative treatments.

In this study for the first time the *in vitro* immunophenotype, embryonic stem cell (ESC) marker expression, clonogenic and proliferation ability and multilineage differentiation potential, including the tenogenic one, of TSPCs and adipose-derived stem cells (ASCs) were compared. Indeed, ASCs have been already well characterized and demonstrated to be potentially useful in musculoskeletal cell-based therapy, but to date very few *in vitro* studies have investigated their *in vitro* tenogenic potential [8-10]. Thus the main aim of this study is to provide further knowledge about the potential use of TSPCs in musculoskeletal tissue regeneration strategies, where our hypothesis is that it could be more advantageous to use this cell source for tendon regenerative purpose in comparison to ASCs.

MATERIALS AND METHODS

TSPCs and ASCs isolation and culture

All the procedures were carried out at Galeazzi Orthopeadic Institute (Milano, Italy) with the Institutional Review Board approval (M-SPER-014.ver7 for use of surgical waste). All the donors gave their written consent to the use of surgical waste material for research purpose. TSPCs were isolated from discarded fragments of semitendinosus and gracilis tendons collected from 7 donors (mean age 33 ± 14 years) who underwent anterior cruciate ligament (ACL) reconstruction; ASCs were isolated from raw lipoaspirates of 7 donors (mean age

47 ± 14 years) who underwent aesthetic liposuction (Table 1). The samples of tendon tissue were minced into small pieces (0.5-0.8 cm), placed in 100 mm Petri dish and covered with control medium (Table 2, CTRL medium). During the first 10 days in culture, tendon cells migrated from tissue and started to proliferate [11, 12]; about 3 weeks later they reached 80-90% of confluence.

ASCs were isolated as previously described [2]. Briefly, adipose tissue was washed with phosphate-buffered saline (PBS) and digested with 0.075% type I collagenase (Worthington Biochemical Corporation, Lakewood, NJ, USA) at 37°C for 30 min. The stromal vascular fraction (SVF) was centrifuged (1200 g, 10 min) and then filtered through a 100 µm nylon cell strainer (Becton, Dickinson and Company, Franklin Lakes, NJ, USA); the collected SVF cells were plated in CTRL medium at 104 cells/cm2 of density and in about 7 days they reached the 80-90% of confluence. TSPCs and ASCs were maintained in culture changing medium every 3 days.

TABLE 1

Characteristic of patients and cellular yield at passage 1

	TSPCs	**ASCs**
Number of patients	N=7 (2 F, 5 M)	N=7 (6 F, 1 M)
Age (years)	33 ± 14	47 ± 14
Grams of tissue for each donor	1.7 ± 0.9 ***	21.5 ± 13.3
Number of cells at P1/grams of tissue	2.2 ± 1.8 X 10^5 *	0.7 ± 0.5 X 10^5

* p<0.05, *** p<0.001 *vs* ASCs

TABLE 2

Composition of differentiation media

MEDIUM	MAIN COMPONENTS	SERUM FBS	SUPPLEMENTS
CONTROL (CTRL)	HG-DMEM 100 U/ml penicillin, 100 µg/ml streptomycin, 0.29 mg/ml L-glutamine	10%	/
ADIPOGENIC (ADIPO) -INDUCTION	CTRL	10%	1µM dexamethasone, 10 µg/mL insulin, 500 µM IBMX (3-isobutyl-1-methyl-xanthine), 200 µM indomethacin
ADIPOGENIC (ADIPO) - MAINTENANCE	CTRL	10%	10 µg/mL insulin
OTEOGENIC (OSTEO)	CTRL	10%	10 nM dexamethasone, 10 mM glicerol-2-phosphate, 150 µM L-ascorbic acid-2-phosphate, 10nM cholecalciferol

CHONDROGENIC (CHONDRO)	CTRL	1%	1 mM sodium pyruvate, 1% ITS+1 (1.0 mg/ml insulin from bovine pancreas, 0.55 mg/ml human transferrin, 0.5 µg/ml sodium selenite, 50 mg/ml bovine serum albumin and 470 µg/ml linoleic acid), 0.1 µM dexamethasone, 0.1 mM L-ascorbic acid-2-phosphate 10 ng/ml TGF-β1
TENOGENIC (TENO)	CTRL	1%	50 ng/ml BMP-12

TGF-β1 : Transforming growth factor β1; BMP-12: bone morphogenetic protein 12

Cell doubling time

For TSPCs and ASCs cell-doubling time (DT) was calculated from passages 2 to 4. The cells were plated at a density of 3 x 103 cells/cm2 in CTRL medium. Fresh medium was supplied every 3 days and at 80–90 % confluence, the cells were splitted cells using trypsin/EDTA (0.5 % trypsin/0.2 % EDTA; Sigma-Aldrich). DT was calculated according to the following formula: DT = CT / ln (Nf-Ni) / ln2,

where CT is the cell culture time (hours), Nf the final number of cells, and Ni the initial number of cells [13].

Fibroblast-Colony Forming Unit Assay (CFU-F)

Clonogenic ability of ASCs and TSPCs from passage 2 to passage 4 was evaluated by colony forming unit assay as previously described [2]. Briefly, cells were plated in six-well plates at low density by limiting dilution (starting dilution: 100 cells/cm^2, ending dilution: 13 cells/cm^2) and cultured in control medium supplemented with 20% FBS for 14 days. Then the cells were fixed with 4% paraformaldehyde solution, and counted after 2.3% Crystal Violet staining (Sigma-Aldrich) for 10 minutes at room temperature. The frequency of CFU-F was established by scoring the individual colonies composed of at least 30 cells and expressed as a percentage relative to the number of the initial seeded cells.

Cell metabolic activity

For both TSPCs and ASCs $1.5x10^4$ cells at passage 4 were seeded in 96-well plates and cell metabolic activity assay was performed at 1, 3 , 7 and 14 days. Briefly, a final concentration of 0.5 mg/mL MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, Sigma-Aldrich) was added to the culture medium and incubated for 4 hours at 37°C. After medium removal, 100% DMSO (dimethylsulfoxide) was added to each well to solubilize the formazan precipitate. The absorbance of this solution was read at 570 nm (VictorX3, Perkin Elmer microplate, Waltham, MA, USA).

FACS analysis

TSPCs and ASCs at passage 4 were detached and washed twice in cold FACS Buffer (PBS w/o Ca/Mg^{2+}, 2% FBS, 0.1% NaN_3). 2.5 x 10^5 cells were incubated with anti-human primary monoclonal antibodies fluorescein isothiocyanate (FITC)-conjugated CD90, CD13, CD31, CD44, CD45, CD166; biotinylated (BIOT)-conjugated CD29, CD34, CD54, CD71, CD105 (all Ancell Corporation, Bayport, MN, USA) and phycoerythrin (PE) conjugated CD73 (Miltenyi Biotec, Bergisch Gladbach, Germany). After incubation, Streptavidin-PE and FITC conjugated goat anti-mouse Ab (Ancell Corporation) were used as secondary antibodies for cells stained with biotinilated antibodies. Background fluorescence was established by negative controls and data on 10000 cell fluorescence events were acquired by flow cytometry using a FACSCalibur flow cytometer and analyzed by CellQuest software (BD Bioscences, San Jose, NJ, USA).

Adipogenic differentiation

Both cell populations at passage 4 were seeded at 10^4 cells/cm^2 and then induced to differentiate toward adipogenic lineage for 21 days using a repeated pulsed protocol, consisting in 3 days of adipogenic induction medium followed by 3 days in adipogenic maintenance medium (Table 2, ADIPO medium). To quantify the lipid vacuoles, cells were rinsed and fixed in 10% neutral buffered formalin for 1 hour, stained with Oil Red O (Sigma-Aldirch) for 15 min, then unstained with 100% isopropanol. Absorbance was read at 490 nm.

Osteogenic differentiation

Cells at passage 4, seeded at 104 cells/cm2 were differentiated into osteogenic lineage by culturing in osteogenic medium (Table II, OSTEO medium). Cells were assessed for alkaline phosphatase (ALP) activity and extracellular calcified matrix deposition [2]. Briefly, after 14 days of differentiation, cells were lysed with 0.1% Triton X-100 (Sigma-Aldrich) and the enzymatic ALP activity was determined by incubating cellular lysates at 37°C with 1 mM p-nitrophenylphosphate (Sigma-Aldrich) in alkaline buffer (100mM diethanolamine and 0.5 mM MgCl2, pH 10.5). The absorbance was read at 405 nm (Victor X3, Perkin Elmer microplate) and the ALP activity was normalized on the total protein content determined (BCA protein assay kit, Pierce Biotechnology, Rockford, IL, USA).

The extracellular calcified matrix deposition was evaluate after 21 days of differentiation. Briefly, the cells were stained with 40 mM Alizarin Red S (pH 4.1, Fluka) for 15 min. The dye was extract with 10% cetylpyridinium chloride monohydrate (CPC, Sigma-Aldrich) in 0.1 M phosphate buffer (pH 7.0) and the absorbance read at 550 nm.

Chondrogenic differentiation

At passage 4, 5.0 x 105 TSPCs and ASCs were centrifuged (250 g, 5 min) to obtain cell pellets. Pellets were cultured in chondrogenic (CHONDRO) medium (Table II) for 21 days. For histological analysis, pellets were fixed for 24 hours in 10% neutral buffered formalin, embedded in paraffin and sectioned at 4 µm. Sections were stained with haematoxylin-eosin (Sigma-Aldrich) and safranin O to evaluate extracellular matrix and glycosaminoglycans (GAGs) deposition [14]. GAGs were

also quantified digesting pellets (16 h, 60°C) in 500 µl of PBE buffer (100 mM Na2HPO4, 10 mM NaEDTA, pH 6.8) containing 1.75 mg/ml L-cystein (Sigma-Aldrich) and 14.2 U/ml papain (Worthington). Samples were incubated with 16 mg/l dimethylmethylene blue (Sigma-Aldrich) and absorbance was read at 500 nm (Perkin Elmer Victor X3 microplate reader). The same samples were used for DNA quantification by CyQUANT Kit (Life Technologies). The amount of GAGs produced for each sample was normalized on DNA content and expressed as µg of GAGs per µg of DNA.

Tenogenic differentiation

Both TSPCs and ASCs populations were seeded at passage 4 and at cell density of 10^4 cells/cm^2. After 7 and 14 days of tenogenic differentiation in inductive medium (Tab II) the expression of tendon-related genes was assessed by RT-PCR.

Gene expression

The total RNA was purified from the cell lysates using the RNeasy Mini kit (Qiagen, Duesseldorf, Germany) and reverse-transcripted to cDNA (5 min at 25 °C, 30 min at 42 °C, and 5 min at 85 °C) using iScriptcDNA Synthesis Kit (Bio-Rad Laboratories, Benicia, CA, USA). Ten ng of cDNA were incubated with a PCR mix (50 °C for 2', 95 °C for 10', 40 cycles at 95 °C for 15 seconds, and 60 °C for 1') containing TaqMan Universal PCR Master Mix and Assays-on-Demand Gene expression probes (Life Technologies, Grand Island, NY, USA) for the following genes: leptin (LEP, Hs00174877_m1), runt-related transcription factor 2 (RUNX2, Hs00231692_m1), collagen type I alpha 1 (COL1A1, Hs01076777_m1), collagen type III alpha 1 (COL3A1, Hs00943809_m1), SRY-box 9 (SOX9, Hs00165814_m1), aggrecan (ACAN, Hs00153936_m1), scleraxis (SCX, Hs03054634_g1), decorin

(DCN, Hs00370385_m1), octamer-binding transcription factor 4 (POU5F1, Hs04260367_gh) and kruppel-like factor 4 (KLF4, Hs00358836_m1). Reaction was performed with Applied Biosystems StepOnePlus (Life Technologies). The fold change in the expression was normalized on the expression of the housekeeping GAPDH gene (glyceraldehyde-3-phosphate dehydrogenase, Hs99999905_m1).

Statistical analysis

Data are expressed as mean ± standard deviation (SD). Normal distribution of values was assessed by the Kolmogorov-Smirnov normality test. Statistical analysis was performed using Student's t-test for data with a normal distribution and Wilcoxon test for data with a non-normal distribution (GraphPad Prism v5.00; GraphPad Software, San Diego, CA, USA). Level of significance was set at $p< 0.05$ (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Correlation between age and each parameter was assessed by Pearson's correlation test.

RESULTS

Undifferentiated TSPCs and ASCs show similar stem cells features

TSPCs and ASCs were harvested from 7 different donors each (Table 1). The mean donors' age of the two cell populations was different, but the large variability within each group make this difference not significant (n.s). The regression analysis performed to assess possible correlation between age and all the parameters evaluated revealed no significant correlation (data not shown).

Although the weight of the starting tendon fragments was significantly lower with respect to lipoaspirates ($p<0.001$), the number of cells at passage 1 normalized per grams of tissue was 2.2 ± 1.8 X 10^5 and 0.7 ± 0.5 X 10^5, respectively for TSPCs and ASCs ($p<0.05$). During passages in culture, TSPCs showed a typical fibroblastic-like morphology and proliferated with a rate similar to the ASCs one (Figure 1a, b).

Indeed, at passage 2 the average doubling time (DT) was 87.0 ± 7.6 hours for TSPCs and 70.8 ± 23.6 hours for ASCs (n.s); then TSPCs and ASCs proliferation rate progressively decreases until reaching at passage 4 an average DT of 176.7 ± 108.1 hours and 190.3 ± 65.8 hours, respectively (n.s.). The clonogenic ability was also similar between the two cell populations along all the passages analyzed; in particular the highest number of colonies was observed at passage 4 for TSPCs (4.4 ± 1.9%) and at passage 3 for ASCs (3.5 ± 1.9%) (Figure 1c). In order to evaluate the *in vitro* cell viability of TSPCs and ASCs, considered an important parameter concerning primary cell survivorship after isolation and during culture, we observed their metabolic activity using MTT assay. No significant differences were also observed in term of cell metabolic activity at passage 4 between TSPCs and ASCs (Figure 1d). The expression of stemness-specific transcription factors KLF4 and POU5F1 of TSPCs was also comparable to that observed for ASCs (n.s.) (Figure 1e).

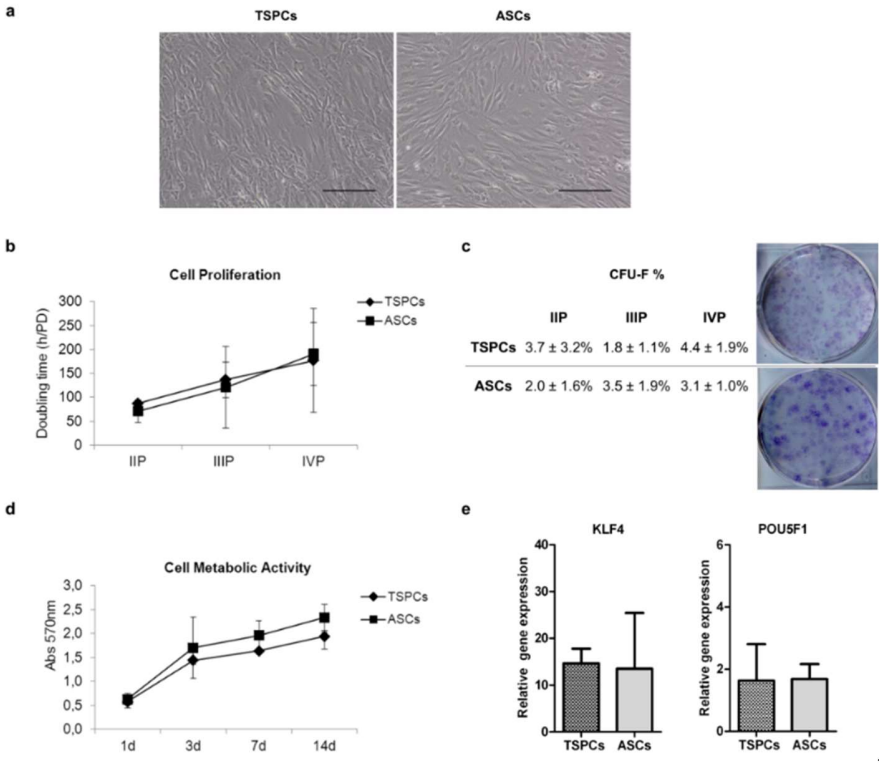

Figure 1: *Features of TSPCs and ASCs. (a) Cell in culture at passage 4 (optical microscopy, 10 X, scale bar 200 µm). (b) Proliferation ability during passages in culture, expressed as mean of doubling times (DT). (c) Percentage of clonogenic cells from passage 2 to passage 4 (scored colonies were normalized on number of seeded cells) and representative pictures of stained CFU-F for both cell population at P4. (d) Cell viability of TSPCs and ASCs at passage 4 at several time points (1, 3, 7, 14 days of culture). (e) Evaluation of KLF4 and POU5F1 gene expression determined by quantitative real-time PCR in both cell populations. Data were normalized on the expression of the housekeeping GAPDH gene.*

The cell size and granularity of both populations were also similar (Figure 2). Moreover, TSPCs were found to possess the characteristic mesenchymal immunophenotypic profile, without any significant difference with regards to ASCs. Indeed they both were highly positive for CD13, CD73, CD90 , CD29, CD44, CD105, CD166 and CD54, and both cell populations did not express hematopoietic markers such as CD31, CD34, CD45 and CD71 (Figure 2).

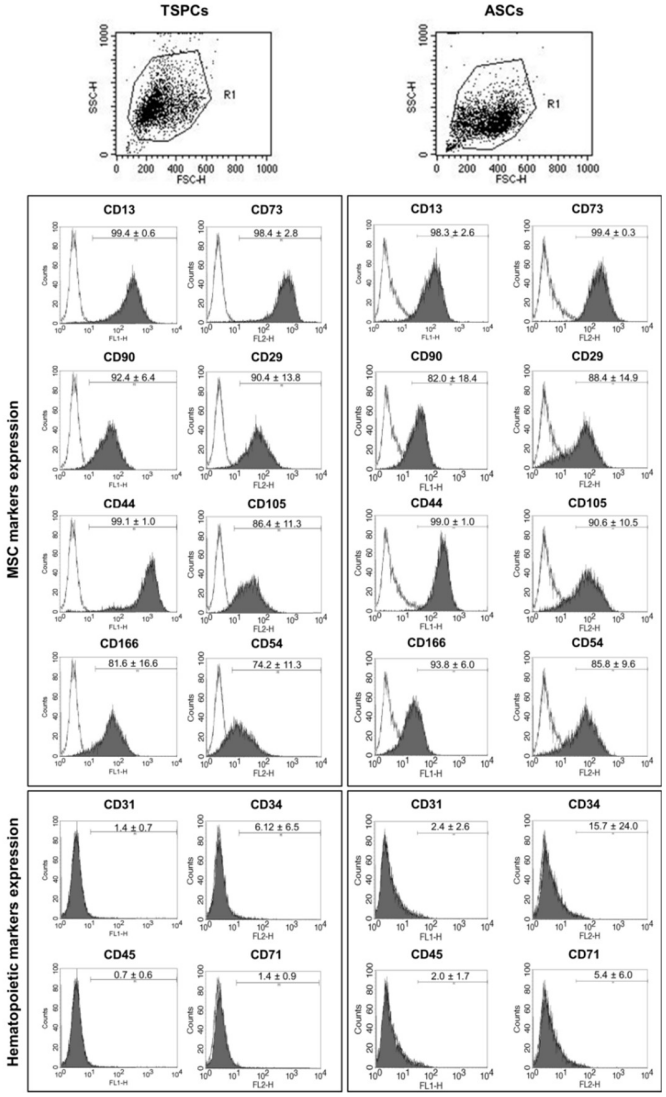

Figure 2: *MSCs surface markers pattern expression. TSPCs and ASCs distribution based on forward scatter (FSC) and side scatter (SSC) and the representative expression of the typical MSCs surface markers and hematopoietic markers for both population at passage 4 (markers are represented as grey histograms and*

isotype control antibodies are represented as white histograms).

TSPCs have a lower adipogenic potential than ASCs

TSPCs cultured for 14 days in adipogenic medium just showed a slight intracellular lipid vacuoles increase respect to cells maintained in non-inductive medium (+40.3%, n.s.) (Figure 3a,b). The lipid vacuoles content observed in TSPCs was significantly lower than that observed in ASCs ($p<0.05$) that, when cultured in adipogenic medium, showed a significant increase in comparison to CTRL ASCs (+136.3%, $p<0.05$). However, both differentiated cell populations expressed significantly higher levels of *leptin* in comparison to their respective CTRL cells ($p<0.05$) and without any difference between them (Figure 3c).

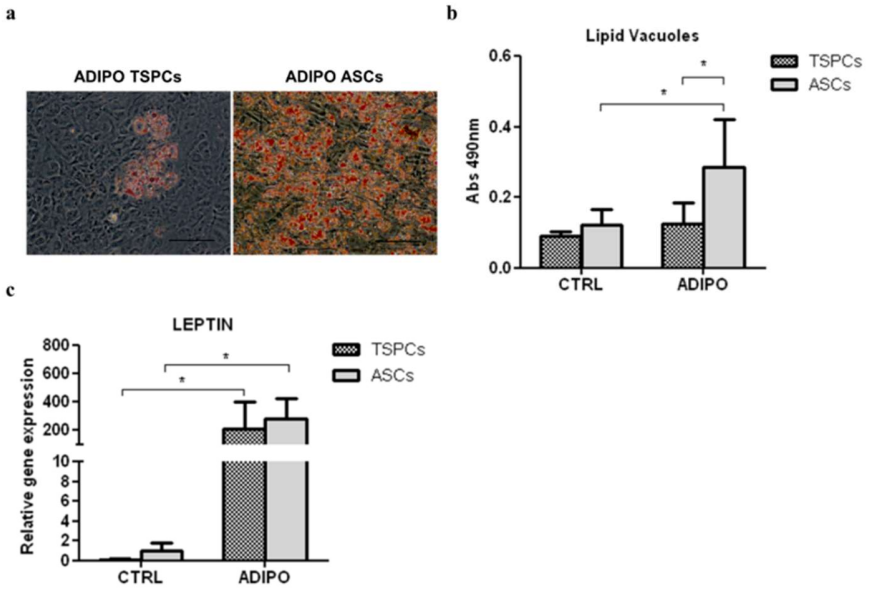

Figure 3: *Adipogenic differentiation. (a) Micrographs of TSPCs and ASCs differentiated towards the adipogenic lineage after Oil Red O staining (scale bars*

*200 µm). (b) Quantification of lipid vacuoles in undifferentiated (CTRL) and adipogenic-differentiated (ADIPO) cells. (c) Gene expression of LEPTIN normalized to GAPDH. Level of significance * p<0.05.*

TSPC osteogenic potential is lower than ASCs one

After 14 days of culture in osteogenic medium, both TSPCs and ASCs showed significant increases in term of ALP activity of 173% and 177%, respectively ($p<0.05$) in comparison to CTRL cells (Figure 4a). Significant up-regulation of *RUNX2* expression was observed in both osteo-differentiated TSPCs (+35%, $p<0.05$) and ASCs (+113%, $p<0.05$) in comparison with the respective CTRL cells. Comparing the two cell types, significantly higher mRNA levels of *RUNX2* were observed in differentiated ASCs with respect to TSPCs (+136% ASCs vs TSPCs, $p<0.01$) (Figure 4b). After 21 days of culture in osteogenic medium, the calcified matrix deposition, as revealed by alizarin red S staining and extraction, was significantly higher in comparison to CTRL cells for both TSPCs and ASCs (+46% and +410%, respectively, both $p<0.05$) (Figure 4c,d). However, the amount of calcified matrix produced by differentiated TSPCs was very similar to undifferentiated ASCs, that after the osteogenic induction produced significantly higher amount of calcified matrix with respect to TSPCs (+ 411%, $p<0.001$). All these data suggest a lower capacity of TSPCs to differentiate toward osteogenic lineage.

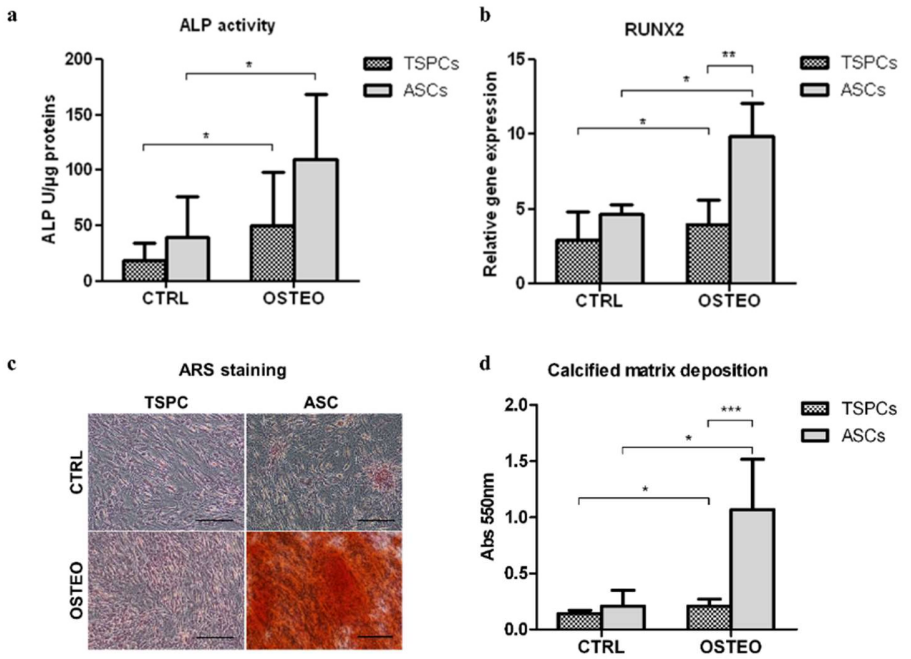

Figure 4: *Osteogenic differentiation. (a) ALP activity determined at 14 days of culture in undifferentiated (CTRL) and osteo-differentiated (OSTEO) cells. (b) Gene expression of RUNX2 normalized to GAPDH at 14 days of culture. (c) Micrographs of CTRL and OSTEO cells stained by AR-S (scale bars 200 µm). (d) Quantification of calcified matrix deposition by AR-S staining and extraction. Levels of significance * p<.05, ** p<0.01, *** p<0.001.*

TSPC chondrogenic potential is higher than ASCs

Both cell populations were cultured in pellet in chondrogenic medium for 21 days. As revealed by the histological evaluation, the pellets cultured in non-inductive medium were smaller and less organized in comparison to the ones cultured in chondrogenic medium for both types of cells (Figure 5a). Moreover,

only the chondro-differentiated pellets of TSPCs showed a marked extracellular matrix production which was intensively stained by safranin-o dye. Chondro-induced TSPCs also showed a significant increase in term of glycosaminoglycan deposition respect to undifferentiated cells (+83%; $p<0.01$); however, undifferentiated ASCs showed significantly higher levels of GAGs in comparison to undifferentiated TSPCs (+96%; $p<0.05$). Both *SOX9* and *ACAN* were strongly up-regulated in chondro-induced pellets (Figure 5c,d) (+251% and +44% for *SOX9,* +1279% and +145% for *ACAN,* for TSPCs and ASCs, respectively) in comparison with CTRL pellets. Moreover, both undifferentiated and differentiated TSPCs showed higher ACAN expression with respect to ASCs (+1208% and +7256% increases for undifferentiated and differentiated TSPCs vs the respective ASCs). However all these differences, due to the wide inter-donor variability, were not significant.

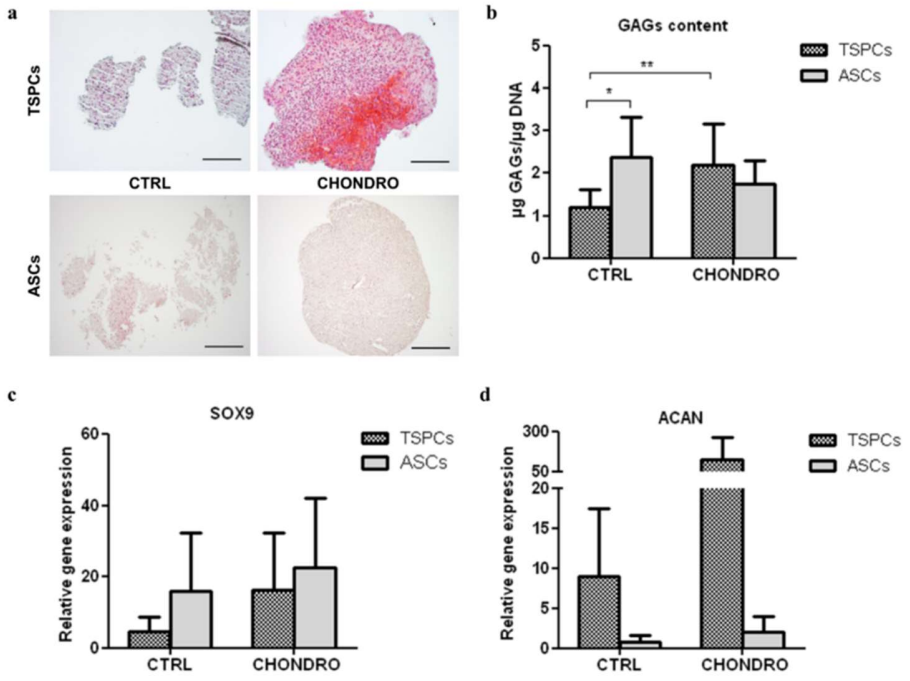

Figure 5: *Chondrogenic differentiation. (a) Histological evaluation using haematoxylin-eosin and safranin staining in undifferentiated (CTRL) and chondro-differentiated (CHONDRO) pellets of both TSPCs and ASCs (scale bars, 200 µm). (b) DNA and GAGs content in TSPCs and ASCs pellets. (c, d) Gene expression of SOX9 and ACAN normalized to GAPDH. Level of significance * p<.05, ** p<0.01.*

BMP-12 increases scleraxis and decorin expression in both TSPCs and ASCs

The BMP-12 dosage used in this study (50 ng/ml) was chosen accordingly to previously experiments carried on ASCs (data not shown).

The gene expression of specific tendon markers was evaluated after 7 and 14 days in undifferentiated and differentiated cells in both cell populations. In undifferentiated cells, SCX was more expressed in TSPCs than in ASCs (+ 461%, $p<0.05$ and +344% at 7 and 14 days, respectively) (Fig.6a, left column). The tenogenic medium was able to induce a further increase in the expression of this transcription factor in both populations; indeed, differentiated TSPCs and ASCs showed higher levels of SCX in comparison with undifferentiated cells both at 7 days (+86% and +89%, respectively) and at 14 days of culture (+50% and +24%, respectively), although these differences were not statistically significant (Fig.6a right column). For what concern the extracellular matrix components, DCN, COL1A1 and COL1A3 gene expression was evaluated. After 7 days of culture in undifferentiated medium TSPCs showed higher levels of DCN in comparison with ASCs (+93%, ns); however, at 14 days of culture this difference was not present anymore as ASCs DCN expression increased with time in culture (Fig.6b left column). Tenogenic medium induced a significant gene up-regulation of this marker in both population already at 7 days of differentiation respect to undifferentiated cells (+50%, $p<0.05$ and +164%, $p<0.01$, respectively). However, after 14 days of differentiation the DCN expression in ASC significantly decreased with respect to the previous time point, whereas TSPCs maintained similar expression level (Fig. 6b right column).

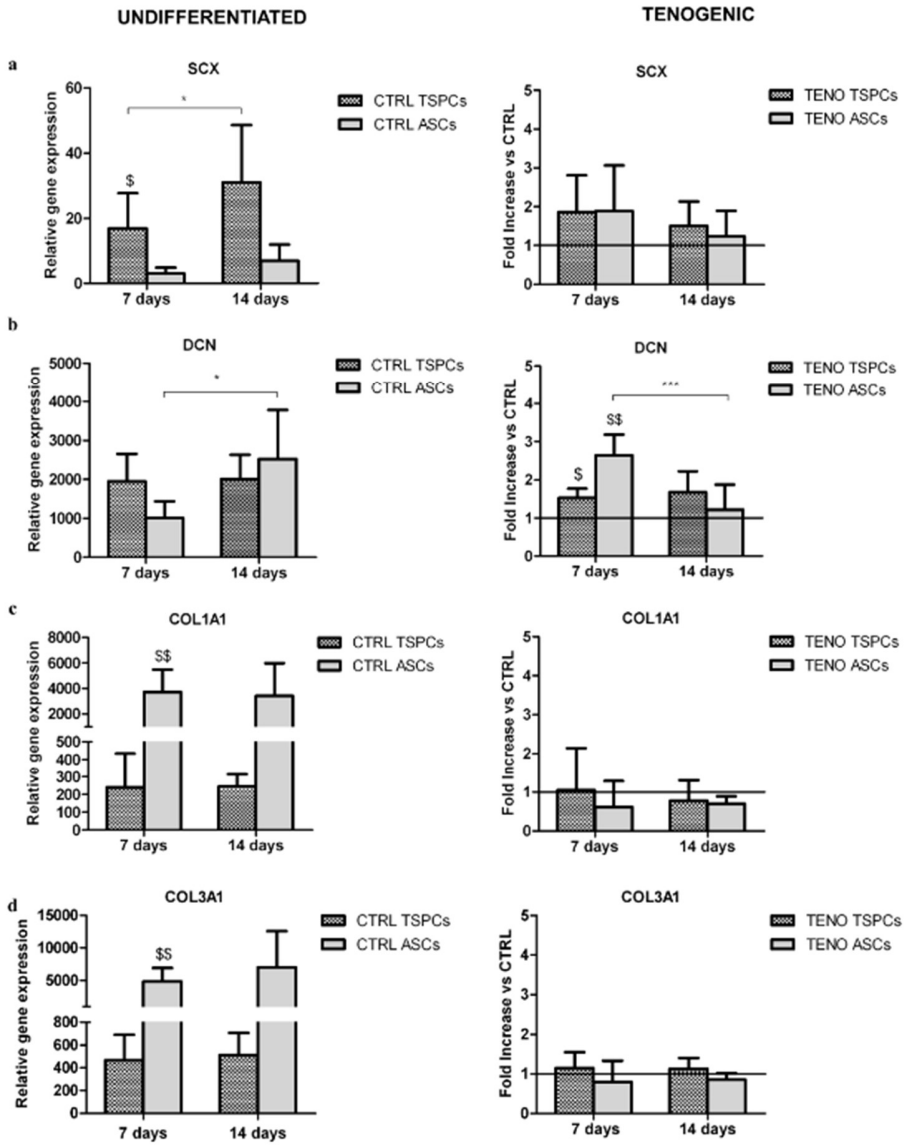

Figure 6: *Tenogenic differentiation. (a-e) Gene expression of scleraxis (SCX), decorin (DCN) and type I and III collagen (COL1A1 and COL1A3) normalized to GAPDH in undifferentiated (CTRL, left panel) and teno-differentiated (TENO, right*

*panel) TSPCs and ASCs at 7 and 14 days of culture. Data for the tenogenic differentiated cells are expressed as average fold increase ± SD respect to undifferentiated ones (fixed line set at 1). Levels of significance * p<0.05, ** p<0.01; ASCs vs TSPCs.*

Unexpectedly, undifferentiated ASCs showed very higher level of COL1A1 and COL3A1 expression respect to TSPCs (+1296%, p<0.01 and% + 1279%, p<0.01, respectively) (Fig. 6c, d left column). Moreover, both cell populations did not show any further increase after tenogenic induction respect to undifferentiated cells (Figure 6c, d right column).

DISCUSSION

To the best of our knowledge this is the first study comparing stem cell properties and multi-lineage differentiation potential of human TSPCs and ASCs. The main finding of our study is that TSPCs and ASCs show a similar cell proliferation, viability and clonogenic ability, as well as stem cell markers expression. On the other hand, although both MSC populations show multi-differentiation potential, some significant differences were observed partially related to their tissue origin, supporting the idea that a given MSC tissue source could be more appropriate for a given clinical situation. Little is known about the tendon biology also for the scarce knowledge of the cell population resident in tendon tissue. Just recently it has been demonstrated that tendon contains stem/progenitor cells that can provide a new tool to study tendon physiology, pathology and possible innovative tendon therapies based on their properties [7]. Indeed, TSPCs are supposed to play a primary role in maintaining the tissue homeostasis and in promoting the

repair after injury and they could be addressed as a new potential therapeutic target. In particular, TSPCs could be the main effector of the *in vitro* biophysical stimuli such as pulsed electromagnetic field and extracorporeal shock waves that have been proposed as promising alternatives for the treatment of tendinopathies [15-17]. Another perspective might involve the use of TSPCs as cell source for cell-based therapy approaches to improve the regeneration of tendon or of other tissues of mesodermal origin such as bone and cartilage. Currently, adipose tissue is considered one of the smartest and most convenient source for MSC isolation due to its wide availability as surgical waste material [18] and for this reason in the last years several cell/tissue banks have focused their activity on its preservation and of the related MSC population. Similarly, as already demonstrated and confirmed by this study, it is possible to efficiently isolate TSPCs from surgical waste tendon fragments, including hamstring tendons that are used in ligament reconstruction. Considering that each year worldwide about 200.000 anterior ligament reconstruction procedures are performed [19], it could be noteworthy to ameliorate the knowledge of the potential of these cells to possibly exploit them for future allogeneic applications, also thanks to their low immunogenicity [20]. In this study we have compared the *in vitro* behavior and the stemness features and multidifferentiation potential of human TSPCs with those of ASCs. The number of cells is crucial for some regenerative medicine applications; in our study the cellular yield of TSPCs was higher with respect to ASCs. Since above all at the first passages in culture the tendon cell population is composed not only by TSPCs but also by terminally differentiated tenocytes, the yield could have been influenced by this factor. However, tenocytes lose their phenotype *in vitro* with time and passages in culture and,

although specific markers have yet to be found, they differ from TSPCs in term of morphology, proliferation potential and expression of stem cells markers such as OCT4 (POU5F1) [20-24]. For all these reasons, the experiments were performed starting from cells at passage 4 (about after 5 weeks of culture), when it was observed that the cell population possessed features peculiar of progenitor cells, including the expression of transcription factor essential for self-renewal maintenance and pluripotency in embryonic stem cells such as POU5F1 and KLF4 [25], and, in agreement with the minimal criteria for defining multipotent mesenchymal stromal cells [26], the expression of specific mesenchymal stem cell markers in standard culture conditions, without any difference with ASCs. Clonogenic assay for fibroblast-like colonies revealed a very similar frequency of cells able to form colony in TSPCs to that observed in ASCs. All TSPC and ASC populations did not show differences in cell metabolic activity suggesting similar cell viability when they are cultured *in vitro*. Moreover, we also observed that TSPCs and ASCs possessed a comparable doubling time of around three days at passage two. These results are consistent with some previous ones, indicating that human and mouse TSPCs proliferated faster than BMSCs [7, 27], which have been demonstrated to possess a lower proliferation ability than ASCs [28, 291], thus explaining the comparable behavior observed between ASCs and TSPCs. The differences in term of multi-differentiation potential of MSCs from different sources have been deeply investigated: previous studies reported higher multi-differentiation potential of mouse and rat TSPCs respect to BMSCs [7, 27]; others found that human ASCs were able to more efficiently differentiate into the adipogenic lineage, but not into the chondrogenic one in comparison to BMSCs [28-30]. In our study also TSPCs and ASCs presented some differences in term of

multi-differentiation potential. In particular, although adipo- and osteo-differentiated TSPCs showed significant increases in the related tissue specific marker expression respect to undifferentiated cells, they possess lower adipogenic and osteogenic potential with regard to ASCs The more pronounced adipogenic potential of ASCs could be attributed to their pre-commitment, whereas the more marked osteogenic potential is also indicated by the very high type I collagen expression that we observed in undifferentiated ASCs, as also already reported in previous studies [31, 32]. Quite unexpectedly, TSPCs seemed to be more susceptible to chondrogenic medium in comparison to ASCs, above all in term of GAG production, although they was already high in undifferentiated ASCs. The more dramatic difference observed between TSPCs and ASCs that underwent the chondrogenic differentiation protocol was the aggrecan gene expression; aggrecan is a proteoglycan, present in cartilage as well as in compressed tendon regions. So, the very high expression of this marker already in undifferentiated TSPCs could be partially related to their tissue origin and consequently to their pre-commitment. Anyway, as articular cartilage has only a poor capacity for self-repair [33], it could be important to further investigate this aspect as hamstrings can be easily harvested during knee surgery, causing minimal additional morbidity to patients which possibly require autologous regenerative treatment for knee concomitant chondral defects. Collectively, all these data confirm that MSCs from different sources can respond differently to stimuli, posing the question whether optimal conditions of differentiation should be properly adapted to the specific MSC type. To achieve a more efficient *in vitro* differentiation protocol, it could be useful to further investigate the cell niche factors, as it is widely accepted that several different environmental factors

contribute to the overall control of stem cell activity. As little is known about the maturation of tendon and its related tendon cell niche factors, consequently few information are available to develop an efficient tenogenic differentiation medium. In previous *in vitro* studies on ESCs, ASCs, and BMSCs, the tenogenic induction has been attempted using a variety of growth factors, including bone morphogenic proteins (BMP-2, -5, and BMP-12, -13, -14, also known as GDF-7, -6, and -5, respectively), insulin-like growth factor-1 (IGF-1), TGF-β1, TGF-β3 [8-10, 34-37]. The influence of BMP-12 on tenogenic differentiation was evaluated in several types of MSCs [37, 38], but only few studies were conducted on ASCs [8, 10], and to our knowledge none of them on human cells. Here, for the first time, we evaluated the effect of BMP-12 on the tenogenic induction of human ASCs and TSPCs. Regarding tendon related markers expression, our results demonstrated that scleraxis, transcription factor involved in tendon development, and decorin, proteoglycan that stabilize and align collagen type I and III fibrils [39, 15, 24], were more expressed in undifferentiated TSPCs compared to undifferentiated ASCs, also in this case probably due to their pre-commitment. However, BMP-12 was able to induce in both populations a strong up-regulation of scleraxis expression, as well as a significant increase of decorin one. On the other hand, tenogenic induction did not affect neither collagen type I nor collagen type III expression in both TSPCs and ASCs, probably suggesting that BMP-12 alone is not able to induce a complete up-regulation of tendon extracellular matrix marker. Our data on ASC tenogenic differentiation confirm what observed by Shen who demonstrated that BMP-12 is capable of inducing tenogenic differentiation in canine and mouse ASCs, but our hypothesis that it could be more advantageous to use TSPCs for tendon regenerative applications

in comparison to ASCs was not confirmed, at least with the protocol here used [8]. Other tenogenic factors, such as GDF-5/BMP-14, should been tested on human ASCs and TSPCs to assess if it is able to induce a more evident tenogenic induction, although previous studies reported that GDF-7/BMP-12 induced the tenogenic differentiation of canine ASCs more efficiently in comparison to GDF-5/BMP-14 [8]. Moreover, the identification of more specific targets could allow to develop new factors able to induce cells toward tendon differentiations more efficiently. Since tendon is a mechanoresponsive tissue, it can be speculated that, together with biochemical stimuli, appropriate mechanical loads would be helpful to improve the tenogenic differentiation of progenitor cells, in particular of TSPCs, as the modulation of physical stress response is part of their physiological role in tendon tissue; indeed external physical stimuli seem to enhance or accelerate the differentiation into tenocyte-like cells [40]. For this reason, further studies are needed to clarify the cell response to mechanical stimuli and also how they are able to influence the fate of MSCs. Another criticism in this research field is that the markers usually used to assess the tenogenic differentiation are not strictly specific for tendon, as they can be also found in other tissues and cells. For this reason the evaluation can be sometimes misleading and thus, to obtain more reliable results, the identification of more specific tendon markers may provide some better insight into the *in vitro* tenogenesis. One of the limitations of the study is the lack of a donor-matched comparison that could have partially reduced the high interdonor variability that often represent a critical point when dealing with primary cells. However, in this case the use of donor-matched cells would have involved ethical issues, since part of the collected tissues should have been intentionally harvested, whereas

in our study all the samples used to isolate cells were surgical waste. Nevertheless, the possibility to obtain data form a donor-matched comparison study could be considered in future studies to better characterize MSCs from different sources. Another limitation of this study is the use of the differentiation protocols that were previously developed and adapted for ASCs, and that could have disadvantaged the TSPC differentiation ability. Moreover, the assessment of just tendon specific gene expression without the related protein expression and the testing of a single growth factor without any mechanical stimulation to induce the tenogenic differentation represent other limitations of this study. Further comparative studies about MSC immunogenicity could be useful to better identify the best cell type for allogeneic use.

CONCLUSIONS

Taken together, our results demonstrated that TSPCs do not significantly differ from ASCs in term of clonogenic ability, proliferation and immunophenotypic profile. In comparison to ASCs, TSPCs are less prone to differentiate into adipogenic and osteogenic lineage using our protocols, but are more able to differentiate into chondrogenic- and tenogenic-like cells, potentially opening the possibility to use them as a new cell type to be used for some regenerative medicine applications in the musculoskeletal field.

FUTURE PERSPECTIVES

Preclinical studies have shown the possibility to exploit the properties of ASCs and BMSCs to manage tendon disorders. Our findings, together with others concerning TSPCs, allow to speculate that tendon could represent in the future

an appealing and sustainable cell source to isolate MSC for musculoskeletal regenerative purposes. Moreover, thanks to the low immunogenicity of MSCs, it could be possible to preserve TSPCs isolated from surgical waste material for allogeneic use, as currently done for other MSC types, including umbilical cord- and adipose tissue-derived ones.

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3.2 Manuscript 2: Tendon Stem/Progenitor Cells are low immunogenic and possess immunomodulatory properties

Viganò M, Perucca Orfei C, Colombini A, de Luca P, de Girolamo L.

This study is ongoing

INTRODUCTION

Human Tendon Stem/Progenitor Cells (TSPCs) are a subpopulation of tendon tissue resident cells that possess Mesenchymal Stem Cells (MSCs) features in terms of immunophenotype, colony formation ability and differentiation potential [Bi Y et al., 2007; Lui PP et al., 2011; Stanco D et al., 2015]. Their physiological role is the maintenance of tissue homeostasis by proliferating and releasing proper biochemical stimuli to influence resident, immune and vascular cells in the tendon [de Girolamo L et al., 2013]. In a pathological environment, their number and activity are reduced [Leone L et al., 2012], causing an imbalance in the tissue homeostasis leading to degeneration, inflammation and pain [McCreesh K and Lewis J, 2013]. They were firstly described in 2007 by Bi and colleagues [Bi Y et al., 2007], and since then a growing interest arose around the possibility to apply TSPCs to regenerative medicine, cell therapies and tissue engineering approaches. It has been demonstrated that TPSCs are suitable

therapeutic target of biophysical treatment, applied in order to increase their activity and to favor endogenous tissue regeneration [de Girolamo L et al., 2013; de Girolamo L et al., 2014]. Moreover, *in vivo* reports described successful transplantation of homologous TSPCs in rats, with improvements in tendon repair [Lui PPY et al., 2016]. Another possible application would be the use of scaffold seeded with TSPCs for the reconstruction of tendon in case of rupture, and also in this case, successful attempts were described [Shen W et al., 2012; Ni M et al., 2013].

The harvesting of TSPCs from patients for autologous application would be quite difficult because of the invasive procedure and the donor site morbidity. By the way, over 100,000 anterior cruciate ligament reconstruction are performed every year in the U.S. [Hughes G and Watkins J, 2006], and the recovery of wasted tendon tissue from these procedures would allow, in a short amount of time, the institution of a cell bank of TSPCs for allogeneic use.

In the view of this kind of allogeneic application, it would be important to assess the possible immunogenicity of human TSPCs. Since the low immunogenicity is a peculiar characteristic of MSCs [Tse WT et al., 2003; Le Blanc K et al., 2003; Klyushnenkova E et al., 2005; Rasmusson I et al., 2003REF immunogenicità MSC] it would be crucial to verify the presence of this feature in TSPCs, in order to confirm the potency of these cells in cell therapy applications. Moreover, MSCs therapies are also based on their immunomodulatory ability, but this aspect is still underinvestigated for what concern TSPCs. Our working hypothesis is that TSPCs possess all the characteristics of proper MSCs, including low immunogenicity and immunomodulatory properties, and we tested this

hypothesis by *in vitro* experiments involving TSPCs and allogeneic peripheral blood leucocytes, and comparing them to ASCs.

MATERIAL AND METHODS

TSPCs and ASCs isolation and culture

TSPCs were isolated from gracilis and semitendinosus tendons deriving from five healthy donors undergoing procedures of anterior cruciate ligament reconstruction with hamstring technique (males, mean age 23.2±8.2 years), under informed consent. Tendon tissue was minced and digested overnight at 37°C with 0.3% collagenase type I. Cells were then seeded at the density of 5,000 cells/cm^2 for expansion in complete medium. Adipose derived stem cells (ASCs) were isolated from subcutaneous lipoaspirates of 1 healthy donor (female; age 53) undergoing liposuction under informed consent. Adipose tissue was washed with PBS several times and then it was digested by incubation with 0.075% collagenase type II for 1 hour. Cells were harvested and seeded at the density of 5,000 cells/cm^2 for expansion in complete culture medium.

PBMCs isolation and culture

Human peripheral blood mononuclear cells (PBMCs) were isolated from 10 mL of blood harvested from four healthy donors under informed consent (2 males and 2 females, mean age 28.75±3.1 years). Blood was diluted 1:1 with PBS, and laid on 10 mL Ficoll Isopaque® (GE Healthcare, Chicago, IL, USA) in a 50 mL tube. After centrifugation, the nucleated cells at the interface between Ficoll and supernatant was recovered by a micropipette. Cells were washed two times with PBS and then maintained in RPMI medium with 10% FBS.

PBMCs reaction

TPSCs or ASCs were seeded in a MW96 at the density of $5x10^4$ cells/well. PBMCs were added $5x10^4$ to each well in direct contact with adherent cells. Plates were maintained at 37°C for 24 and 72 hours, and then samples were harvested for ELISA and BrdU assays, respectively.

Inflammatory stimulation of* PBMCs *culture

After isolation, 100 ng/ml of anti-CD3 and anti-CD28 (Mabtech, Nacka, Sweden) were added to PBMCs and maintained throughout the experiment, until sample harvesting after 24 or 72 hours, in order to induce T cell activation [Trickett A and Kwan YL, 2003]. As described above, $5x10^4$ activated PBMCs were maintained in co-culture with $5x10^4$ TSPCs to test immunomodulatory ability. One population of ASCs was included in the study in the same conditions as control.

PBMCs proliferation

BrdU solution was added in each well at a final concentration of 10 µM. Then, after 8 hours of incubation, the culture plate was centrifuged at 300 xg for 10 minutes. After medium removal, the wells were dried at 40°C for 1 hour. BrdU Cell Proliferation Assay (Roche, Basel, Switzerland) was used following manufacturer's instruction. Briefly, an enzyme-conjugated anti-BrdU antibody was added in each well and incubated for 1 hour at room temperature. Then, a chromogenic substrate was added in each well and absorbance @370 nm was monitored every 10 minutes for up to 1 hour with a spectrophotomer (Victor X3, Perkin Elmer, Waltham, MA, USA).

ELISA assay

Elisa assay for IL-2 was performed according to manufacturer's instructions (Mabtech). Assay range of detection was 4-400 pg/mL.

RESULTS

TSPCs are not immunogenic

Different populations of TSPCs (n=5) were plated in presence of allogeneic PBMCs. The proliferation of PBMCs was not increased by the co-culture in any sample, as well as in the ASCs co-cultured PBMCs, and they remained inactivated (Fig. 1A). As a confirmation, PBMCs did not produce IL-2 in any co-culture condition (Fig 1B).

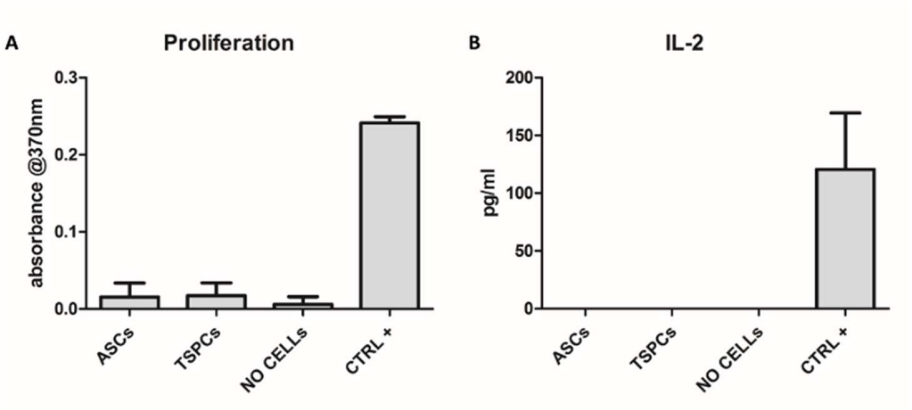

Figure 1. PBMCs cultured in presence or absence of MSCs from tendon or adipose tissues. Proliferation at 72h of incubation (A) and production of IL-2 after 24h of incubation (B). Positive control is represented by PBMCs activated by anti-CD3/anti-CD28 and cultured in absence of MSCs.

TSPCs reduce activation of allogeneic PBMCs stimulated with anti-CD3 and anti-CD28

PBMCs activated with specific T cell stimulation demonstrated an increase in both proliferation and expression of the proinflammatory cytokine IL-2. The co-culture of TSPCs or ASCs with PBMCs did not prevent the proliferation induced

by anti-CD3/CD28 (Fig. 2A), but it completely inhibited the release of IL-2 (Fig. 2B).

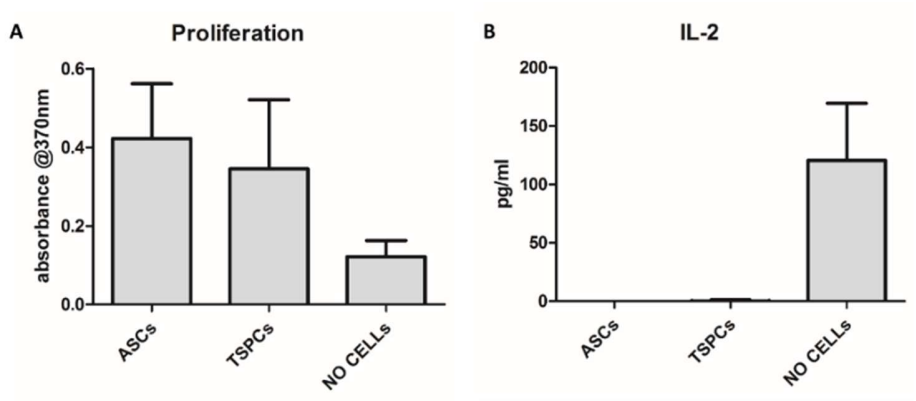

Figure 2. PBMCs stimulated with anti-CD3/anti-CD28 in presence or absence of MSCs from tendon or adipose tissues. Proliferation at 72h of incubation (A) and production of IL-2 after 24h of incubation (B).

DISCUSSION

Mesenchymal Stem Cells represent the most powerful tool in regenerative medicine approaches, thanks to their proliferative, differentiation, trophic and immunomodulatory abilities. The emerging evidence of tissue specific stem/progenitors cells needs in non-bone tissue engineering, due to the propensity of MSCs to differentiate toward an endochondral ossification pathway [Somoza RA et al., 2014], raised great interest in the capability of Tendon derived Stem/Progenitor Cells. Indeed, this is related to the low efficacy of current treatment for tendinopathy, leading to the pursuit of innovative treatment, such as cell-based therapies. Moreover, the high propensity of MSCs from adipose tissue and bone marrow to differentiate towards an endochondral

ossification pathway underlined the risks of their use in tendon pathologies, since ossification often occurred when these approaches were attempted [Harris MT et al., 2004; Lui PP et al., 2012]. Unfortunately, donor site morbidity in the case of TSPCs harvesting would be a great obstacle to their autologous clinical application. Then, it is crucial to define TSPCs immunogenicity in the view of possible rejection after transplantation, as well as their immunomodulatory ability, to assess all the aspect of their therapeutic potential. Indeed, rat TSPCs nonimmunogenicity was reported in few *in vivo* studies, based on their lack in the expression of MHC-II and the systemic or *in situ* evaluation of the inflammatory reaction following transplantation [Lui PPY et al., 2014; Shen W et al., 2012]. For what concern the human cells, just one report explored the effect of decellularization before tendon allograft transplantation, identifying a beneficial effect in terms of lower immune response [Raghavan SS et al., 2012]. Nevertheless, this experiment did not assess specifically TSPCs immunogenicity, whereas it comprise all cell type and antigens within the entire transplanted tissue, and the reaction was observed in a rat recipient. Thus, the topic of human TSPCs immunogenicity and immunomodulatory activity is extremely under investigated. Our results confirm the hypothesis that TSPCs are non- or low-immunogenic, since they are not able to induce an activation of peripheral blood mononuclear cells when directly co-cultured. Indeed, TSPCs in direct contact elicited neither proliferation nor production of inflammatory mediators in the PBMCs.

For what concern the immunomodulatory effect of TSPCs, they performed similarly to ASCs. Indeed, ASCs ability in modulating immune cells response to inflammatory stimuli is well known [Murphy MB et al., 2013]. When PBMCs were

stimulated with antibodies anti-CD3/CD28, a condition well mimicking physiological activation by antigen presenting cells [Trickett A and Kwan YL, 2003], they become activated, starting to proliferate and secreting inflammatory mediators. In this condition, cytotoxic T cells are the main component of the immune reaction, as confirmed by the production of IL-2. The presence of TSPCs blocked the production of this cytokine, while no effect was observed on the proliferation of the PBMCs. Indeed, the main role of MSCs in immunomodulation is the shift from an inflammatory phenotype, towards a modulatory one [Iyer SS and Rojas M, 2008; Spaggiari GM et al., 2009; Selmani Z et al., 2008]. In this context, the uncoupling between proliferation and pro-inflammatory cytokine production allow the speculation of a similar effect of TSPCs on PBMCs in direct contact.

The present work is not complete, since the assessment of the expression of HLA class I and II in TSPCs is still ongoing. This represent a crucial point in the definition of the immune-profile of TSPCs and the mechanism of interaction between them and the cells of the immune systems. Moreover, a larger panel of cytokines, as well as different activation protocols will be analyzed to evaluate the effect of TSPCs on immune cells diverse from cytotoxic T cells.

At the present time, the results obtained in this study support the use of TSPCs in allogeneic Regenerative Medicine applications, and also provide a proof of concept of their use with the aim of control the immune reaction within a pathological environment.

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3.3 Manuscript 3: Comparison of cartilage, disc cells and MSCs stemness features and response to inflammation: a two-step approach based on ASCs secretome and resident progenitors stimulation

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SUMMARY

A reservoir of mesenchymal progenitors is present in the cartilaginous tissues. The stemness features and the behavior of cartilage (CCs), and intervertebral disc (IVDCs) cells in an inflammatory environment in comparison with BMSCs and ASCs was not fully investigated yet.

CCs and IVDCs formed colonies, express stemness markers, osteo-differentiate as well as the MSCs, but showed no appreciable signs of adipo- and, as expected, a better chondro-differentiation.

In an inflammatory environment, all cells showed high expression of catabolic and angiogenic markers and low TGFβ expression. A higher release of the anti-inflammatory IL-1Ra was observed only in IVDCs.

MSCs showed the highest basal levels of IL-6 and anabolic markers. ASCs revealed the best anti-inflammatory behavior with the highest basal IL-1Ra levels and a secretome able to modulate the inflammation in CCs and IVDCs.

The stimulation of cartilage and disc progenitors in an uninflamed environment, could enhance a more physiological tissue repair.

INTRODUCTION

The study of adult stem cells in tissues considered terminally differentiated and having a limited self-renewal ability is a research field partially unexplored and

of growing interest (Caplan 2015). A subpopulation of human cartilage (CCs) (Barbero 2003; Alsalameh 2004; Fickert 2004; Thornemo 2005; Thalleden 2006; Hiraoka 2006; Pretzel 2011; Jiang 2015; Jiang 2016; Oda 2016), nucleus pulposus (NPCs), annulus fibrosus (AFCs) and cartilaginous endplate (EPCs) cells (Brisby 2013; Wang 2016; Liu 2011), showing phenotypic plasticity and a stem-like immunophenotype, was reported to be present in degenerated tissues which share some pathophysiological features such as adult hyaline articular cartilage (AC) of joint and fibrocartilaginous intervertebral disc (IVD).

In general, the role of mesenchymal stem cells (MSCs) in the maintenance of tissue homeostasis resides in their ability to secrete bioactive molecules in inflammatory condition, promoting trophic and immunomodulatory effects. In particular, MSCs arising from perycites help the damaged tissue to create a "regenerative microenvironment" through the stimulation of angiogenesis, the promotion of the mitosis of tissue specific progenitors and the inhibition of cell apoptosis and tissue fibrosis (Caplan 2016). The avascular nature of AC and IVD do not allow MSCs migration at the damaged site during the acute phase of an inflammatory process. During the treatment of damaged cartilaginous tissue, for example, the strategy to practice microfractures (Wright 2009) during surgery was employed to recruit *in situ* bone marrow mesenchymal stem cells (BMSCs) from subchondral bone. Nevertheless, although BMSCs are considered the more promising stem-source for musculoskeletal tissue engineering approaches, they are not able to replace hyaline articular cartilage, preventing the formation of hypertrophy and fibrosis, probably due to their intrinsic endochondral ossification potential (Somoza 2014).

In this context, adult tissue specific progenitors present in AC and IVD represent an alternative potential reservoir of specialized cells for tissue healing and homeostasis. Their plasticity make these cells similar to the MSCs, but their trophic and immunomodulatory role, to our knowledge, was not assessed anymore.

Based on these evidences, one of the main question is why tissue specific progenitors are not able to respond in an effective way to inflammatory injuries and to promote tissue healing and local homeostasis in AC and IVD?

Aim of this study is, at first, to characterize human CCs from joint articular cartilage, and cells from different regions of the human IVD, the nucleus pulposusu (NP), annulus fibrosus (AF), cartilagineous endplate (EP) for their stemness features in comparison with BMSCs and adipose-derived mesenchymal stem cells (ASCs). The second purpose of this work is to investigate the response of these cells to an inflammatory environment in term of ability to produce trophic factors and modulate the inflammation. Finally, due to the emerging role of the MSC-derived secretome as promising anti-inflammatory/trophic cell-free approach for osteoarthritis (OA) treatment (Ruiz 2016), we evaluated the composition of this biological agent.

These results will be useful to identify new strategies for the treatment of cartilaginous and disc lesions, starting from the use/stimulation of resident and highly specialized cells for tissue healing and homeostasis maintenance.

RESULTS

IVDCs formed colonies better than BMSCs and CC. IVDCs and CCs express stemness markers as well as the MSCs and showed a lower osteo-, no appreciable signs of adipo- and a better chondro-differentiation.

IVDCs showed a higher clonal potential in comparison with BMSCs both at P1 and P3 and with CCs at P1; on the contrary no differences were observed between IVDCs and ASCs in the CFU-F ability. Higher clonal ability was showed by ASCs in comparison with BMSCs and CCs at P1. A decrease in the CFU-F ability was observed for ASCs from P1 to P3, that was lower in comparison with IVDCs ability. Form P1 to P3 only CCs showed an increase in clonogenic ability that became higher in comparison with BMSCs ability. Figure 1 a shows CFU-F assay results.

All the analyzed cells osteo-differentiated both at P1 and P3. ASCs showed a better osteo-differentiation potential in comparison with CCs and IVDCs both at P1 and P3 and in comparison with BMSCs at P3. At P1 BMSCs showed a higher osteo-differentiation potential in comparison with IVDCs, but at P3 they showed a reduction in this potential. Figure 1 b shows data concerning calcified matrix deposition at P1 and P3.

At P1 all the cells, with the exception of the ASCs, differentiated toward the chondrogenic phenotype and, as expected, CCs, NPCs, AFCs and EPCs showed higher levels of GAGs deposition in comparison with MSCs.

At P3 all the cells, with the exception of the EPCs, differentiated toward the chondrogenic phenotype. Higher levels of GAGs deposition were observed in CCs and NPCs in comparison with MSCs and in AFCs in comparison with ASCs. As observed for the EPCs, from P1 to P3 also CCs and AFCs showed a decrease of GAGs deposition.

Only ASCs and BMSCs at P1 (Figure 1 d) and ASCs at P3 (data not shown) showed appreciable and comparable signs of adipo-differentiation; a tendency was observed only for CCs at P1.

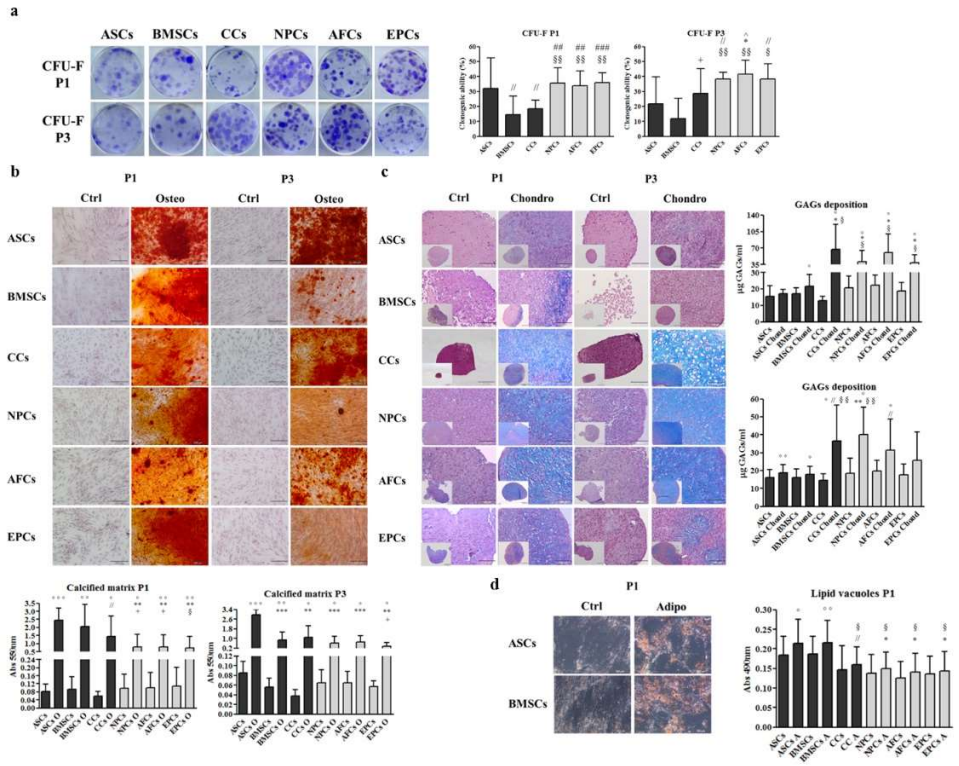

Figure 1. Clonogenic ability, osteo-chondro-adipo differentiation potential. *a) Clonogenic ability. b) Osteo-differentiation potential. c) Chondro-differentiation potential. d) Adipo-differentiation potential. Adipose(ASCs)-, bone marrow(BMSCs)-derived mesenchymal stem cells and cartilage chondrocytes (CCs) obtained from the same 8 donors (dark bars) and of nucleus pulposus (NPCs), annulus fibrosus (AFCs), cartilaginous endplate (EPCs) cells obtained from*

*the same 8 donors (grey bars). * comparison with ASCs, § comparison with BMSCs, # comparison with CCs, // tendency for comparison with ASCs, + tendency for comparison with BMSCs, ^ tendency for comparison with CCs, ° comparison with the treatment. Data are represented as mean ± SD.*

For what concern the immunophenotype of the analyzed cells, as expected, the MSCs were negative for CD14, CD34, CD45, CD71 and positive for CD44, CD73, CD90 CD105, CD151, CD166 expression. BMSCs were positive also for CD146, on the contrary ASCs, CCs and IVDCs were negative for the expression of this marker. CCs and IVDCs showed a substantial similarity with MSCs, showing negativity or positivity for the expected surface stemness markers. Notably, CD14 showed higher positivity in IVDCs in comparison with ASCs and CD34, CD45 showed higher positivity in CCs and IVDCs in comparison with MSCs. In addition, a lower expression of CD90 was observed in MSCs in comparison with CCs and IVDCs. Finally, a peculiar lower CD166 expression was observed in CCs in comparison with MSCs and IVDCs.

Data concerning surface markers expression are shown in Table 1.

	CD14	CD34	CD45	CD71	CD44	CD73	CD90	CD105	CD146	CD151	CD166
ASCs	3.15±2.14	1.19±0.65	0.37±0.45	11.78±7.03	99.63±0.30	99.46±0.94	54.36±25.89	95.06±3.26	9.36±7.73	85.74±11.41	59.36±23.17
BMSCs	5.19±3.19	0.52±0.29//	0.20±0.16	12.00±11.22	99.65±0.27	99.81±0.15	42.71±15.82	96.76±1.17	36.34±15.11*	82.96±15.51	78.35±11.77//
CCs	4.29±2.43	3.28±2.34*§	1.66±1.09*§§	6.15±1.82//	97.13±4.26//+	99.75±0.17	92.51±10.94*§§	93.76±5.29	6.78±4.70§§	95.44±0.69//	28.01±19.27//§§
NPCs	6.81±2.64*^	2.67±2.43+	2.28±1.94//§	14.49±10.85^	98.34±3.01	99.60±0.40	94.45±2.92**§§§	87.03±14.39	5.85±2.94§§	95.07±3.97	65.07±20.94#
AFCs	11.07±6.80**#	2.71±2.03//§	2.69±1.43**§§	15.87±10.26^	98.50±1.63+	99.46±0.70	94.22±5.68**§§§	94.04±7.28	3.35±1.44//§§§^$	87.87±16.94	73.00±19.14##
EPCs	15.71±11.55*+^	3.33±2.33//§	3.89±2.14**§§^	13.44±11.16	96.41±4.86+	99.73±0.17	94.58±3.55**§§§	91.36±5.27+	5.23±2.44§§	88.86±8.84	68.79±26.63#

Table 1. Immunophenotype. *a) Representative expression of the typical MSCs surface markers pattern in all the analysed cells. b) Percentage of positive cells for the whole panel of surface markers tested at P4. * comparison with ASCs, § comparison with BMSCs, # comparison with CCs, // tendency for comparison with ASCs, + tendency for comparison with BMSCs, ^ tendency for comparison with CCs, $ tendency for comparison with NPCs. Data obtained from 5 donors. Data are expressed as mean ± SD.*

High expression of catabolic and angiogenic markers and low TGFβ expression in inflamed CCs, IVDCs and MSCs

The treatment with IL-1β induce a significant increase in the expression of the catabolic enzymes MMP-1, MMP-3 and MMP-13, in all the analyzed cell types (Figure 2 a, b, c). In particular, MMP-1 expression resulted enhanced by 15- to 45-fold in all cell types, while MMP-3 resulted increased by 10-, 600- and 700-fold in ASCs, BMSCs, and CCs respectively; in the IVDCs these increases range between 100- and 300-fold. MMP-13 expression increments were 50- and 30-fold for what concern ASCs and BMSCs, respectively, but only 4 times in inflamed CCs with respect to untreated controls. IL-1β increased the expression of MMP-13 by 20-fold in IVDCs. Interestingly, this treatment enhanced the expression of the pro-angiogenic factor VEGF in BMSCs, ASCs and CCs, while for what concern IVDCs, this increment is significant in AFCs only (Figure 2 d).

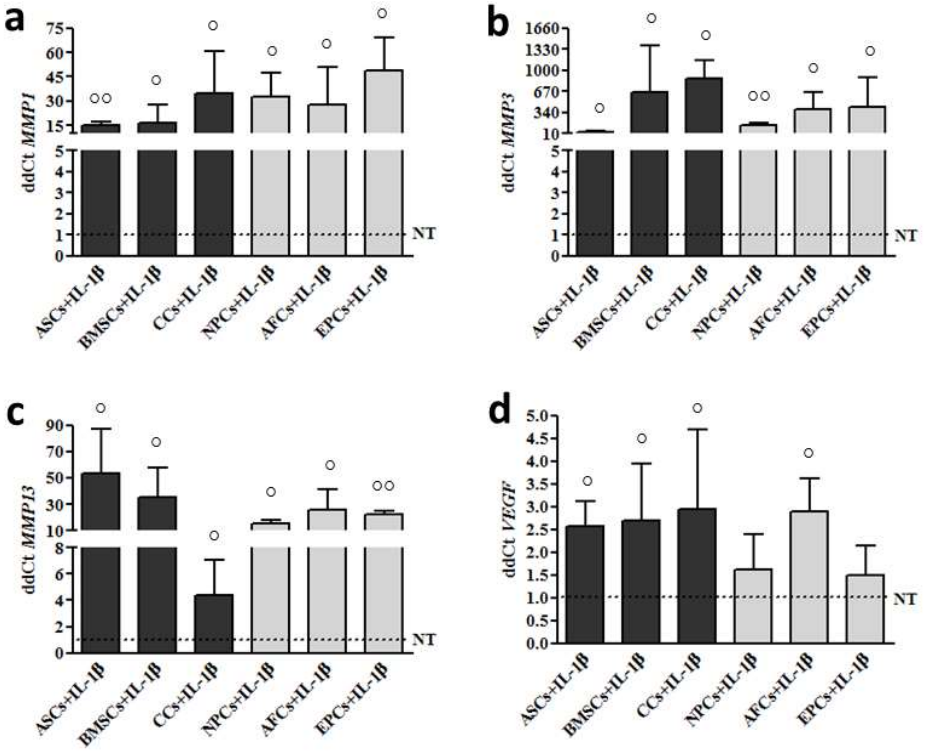

Figure 2. Catabolic response to pro-inflammatory stimulus. *° comparison with the treatment. Data are expressed as mean ± SD.*

Inflammation does not increase anabolic markers production in CCs and MSCs, and ASCs express the higher basal level of metalloproteinases inhibitors

The gene expression of the cartilage anabolic factor TGFβ was reduced by IL-1β treatment in all analyzed cell populations (Figure 3 a). The inflammatory environment, except for what concerns BMSCs, did not affect the expression of TIMP-1, while TIMP-3 significantly decreased in presence of IL-

1β in ASCs, AFCs and EPCs (Figure 3 b, c). Nevertheless, the basal level of this marker resulted higher in ASCs and BMSCs with respect to CCs and IVDCs (Figure 3 d).

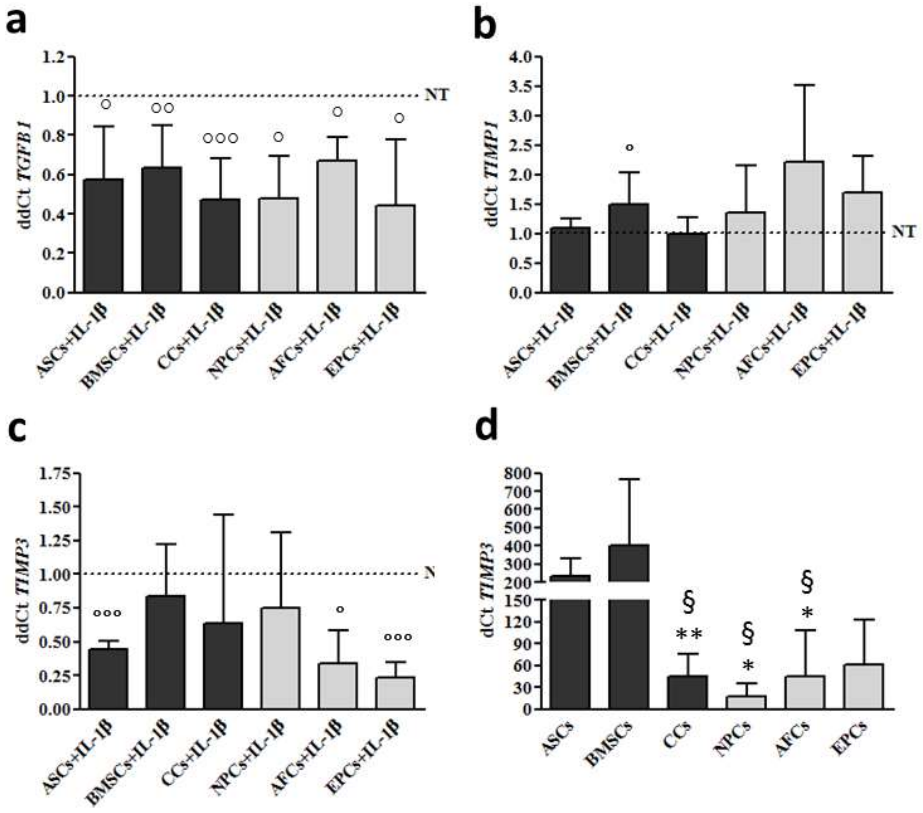

Figure 3. Anabolic response to pro-inflammatory stimulus and basal level of TIMP-3 expression. ** comparison with ASCs, § comparison with BMSCs, ° comparison with the treatment. Data are expressed as mean ± SD.*

ASCs showed the highest basal levels of anabolic markers and a secretome able to modulate the inflammation in CCs and IVDCs.

The production of the pro-inflammatory marker TNFα was not affected by the treatment with IL-1β for all cell type, but it resulted more expressed at basal level in CCs with respect to ASCs and BMSCs (Figure 4 b). Similarly IL-1RA was not induced by IL-1β treatment in any cell type, but ASCs demonstrated to possess a higher basal expression of this anti-inflammatory marker, in both inflamed and non-inflamed environment (Figure 4 c). On the contrary, IL-6 production is directly induced by this kind of inflammatory stimulation in all analyzed cell type, but the IVDCs demonstrated a lower basal expression of this cytokines with respect to ASCs, BMSCs and CCs (Figure 4 a).

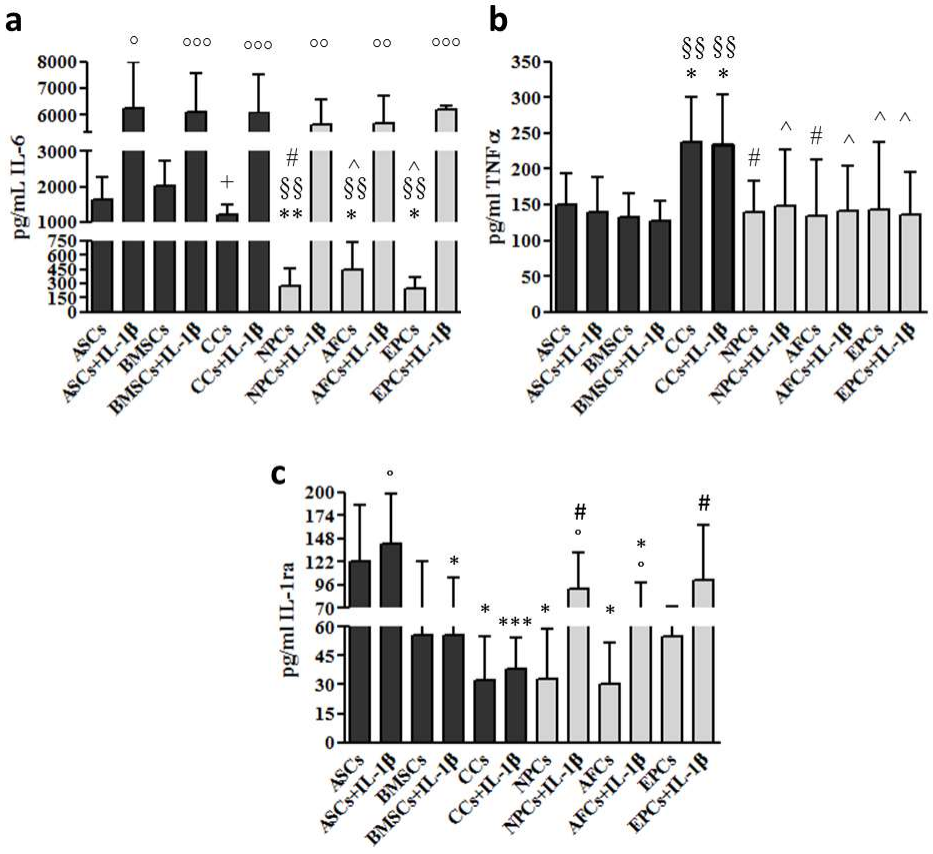

Figure 4. Production of pro- and anti-inflammatory mediators. ** comparison with ASCs, § comparison with BMSCs, # comparison with CCs, + tendency for comparison with BMSCs, ^ tendency for comparison with CCs. Data are expressed as mean ± SD.*

DISCUSSION

Highly heterogeneous populations of multipotent mesenchymal progenitors, displaying the adipo-osteo-chondro-differentiation ability are present in the

superficial and middle zones of the human adult normal and OA AC (Barbero2003; Alsalameh 2004; Thornemo 2005; Pretzel 2011) and in the three main anatomical regions of the human degenerated IVD (Wang 2016). A relative enrichment of these multipotent mesenchymal progenitors chondrocytes during *in vitro* cultivation (Fickert 2004; Jiang 2016) and a variable percentage in OA and normal cartilage (Hiraoka 2006; Oda 2016), without a reduction of the chondrogenic performance related to the pathology (Oda 2016) was reported. In addition, these chondro-progenitors and AF and CEP progenitors showed greater chondrogenic potential in comparison with BMSCs (Jiang 2016; Wang 2016; Liu 2011) and were efficient in cartilage formation *in vivo* and in repairing large knee cartilage lesions in patients (Jiang 2016).

Tissue specific progenitors present in AC and IVD were generally isolated by clonal ability and characterized trough multi-lineage differentiation and surface markers assessment, such as CD105 (Pretzel 2011; Alsalameh 2004; Bernstein 2013), CD166 (Pretzel 2011; Alsalameh 2004; Fickert 2004) and CD146 (Jiang 2016) for chondro-progenitors.

In this study cells obtained from AC and IVD were not selected for their clonogenicity or for specific immunophenotypes, but the heterogeneous population of cells obtained from these tissues were characterized at P1 and P3and maintained in culture without growth factors. We observed that all the analyzed cells were able to form colonies and to osteo- and chondro-differentiate even after expansion. In comparison with the MSCs, they have a lower osteo- and a no appreciable adipo- differentiation, but, as previously showed by other authors (Jiang 2016; Wang 2016; Liu 2011), they better chondro-differentiate. After expansion all the cells were negative for the

expression of hematopoietic markers and strongly positive for other classical MSCs markers (CD73, CD44, CD105, CD151). A great variability and low expression was observed for CD146 (also in ASCs, only BMSCs expressed this markers a slighter higher levels), CD90 was more expressed in CCs and IVDCs in comparison with MSCs, CD166 has its lower expression in CCs and is variable expressed in the other cells. The heterogeneity of the cell populations, the inter-donor variability and the effect of the *in vitro* expansion, this aspect has been already observed for the BMSCs isolated from the femoral head (Etheridge 2004), probably account for the observed variability of the expression of some surface markers.

Based on these evidences we can conclude that CCs and IVDCs are a mixed population of terminally differentiated cells and progenitors with MSCs-like features, but showing a preferential commitment towards the chondrogenic lineage, as expected. The in situ stimulation of these highly specialized progenitors could enhance a more physiological tissue repair and homeostasis maintenance in AC and disc. Nevertheless these tissues are known as having a poor heling ability in pathological conditions. It remains already to assess if this is related to a deficiency in the metabolic response of the resident (progenitor) cells or to the presence of an inflammatory disadvantageous environment, unbalanced toward the tissue catabolism, and resident cells unable to restore the tissue homeostasis.

The inflammatory processes, in particular IL-1β mediated (Le Maitre 2007, Daheshia 2008), are important components of OA and disc degeneration. For this reason we set-up an *in vitro* model of inflammation IL-1β mediated and evaluated the cellular response in terms of known catabolic/inflammatory and

anabolic/anti-inflammatory markers production (Lieberthal 2015). The pro-inflammatory stimulus determined an increase of catabolic and angiogenic mediators, of the pro-inflammatory IL-6 and a decrease of the trophic TGF β in all the cells. CCs showed the worse anti-inflammatory behavior, with the highest basal levels of TNFα release. Interestingly, only IVDCs showed an attempt to respond to the inflammation by increasing the IL-1Ra release and showing sometimes detectable levels of IL-10.

The immunomodulatory and anti-inflammatory effects of the MSCs are well known (Zhao 2010; Pers 2015), accordingly we observed in basal condition an anti-inflammatory behavior of these cells, showing higher TIMP1 and TIMP3 expression and higher IL-1Ra levels produced by ASCs. Nevertheless, in our *in vitro* model, MSCs responded to the inflammatory environment by producing catabolic and angiogenic mediators, without an anabolic balance. For this reason, we proposed an alternative cell-free approach based on ASCs secreted bioactive factors to potentiate the response to the inflammation of the CCs and IVDCs.

EXPERIMENTAL PROCEDURES

Cell isolation and expansion

The study was approved by the Institutional Review Board and specimens were collected with patient informed consent.

NP, AF and CEP from lumbar IVD of 8 patients affected by discopathy were harvested during discectomy. AC, bone marrow and subcutaneous adipose tissue were collected during total hip arthroplasty of the femoral head of 8 OA patients. CCs (Lopa 2013a), NPCs, AFCs (Colombini 2015), EPCs (Lopa 2016), ASCs (Lopa 2011) were isolated by enzymatic digestion as previously described.

Bone marrow was washed in PBS, centrifuged, and BMSCs were selected for plastic adherence (Lopa 2013b).

CCs, NPCs, AFCs and EPCs were cultured in control medium consisting of 4.5 mg/mL high Glucose DMEM (HG-DMEM, Life Technologies, Carlsbad, CA, USA) supplemented with 10% FBS (Lonza, Basel, Switzerland), 0.29 mg/mL L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 10 mM HEPES, 1 mM sodium pyruvate (all reagents from Life Technologies).

ASCs and BMSCs were cultured in □MEM supplemented as reported above; 5 ng/ml FGF-2 (Peprotech) was added to preserve the chondrogenic potential (Liu 2012; Solchaga 2005).

For all cells the seeding density was $5x10^3$ cells/cm^2.

Cells were expanded for one passage (about 10 days, indicated as P1) and three passages (about 30 days, indicated as P3).

Clonogenic ability

A colony-forming unit-fibroblast (CFU-F) assay was performed to assess the clonogenic ability of cells (Lopa 2014). After 14 days, cells were stained with Gram's crystal violet (Sigma-Aldrich, St. Louis, MO, USA). CFU-F frequency was established by counting the colonies and expressing them as a percentage relative to the number of seeded cells.

Immunophenotyping

For flow cytometry, 2.5 x 10^5 cells were incubated with anti-human primary monoclonal antibodies: CD14-FITC, CD34-biotinylated, CD44-FITC, CD45-FITC, CD71-biotinylated, CD105-biotinylated, CD166-FITC (all from Ancell Corporation,

Bayport, MN, USA), CD90-FITC and CD73-PE (from Miltenyi Biotec, Bergisch Gladbach, Germany), and CD151 (R&D Systems, Minneapolis, MN, USA).

Cells stained with biotinylated antibodies were incubated with streptavidin-PE (Ancell Corporation), whereas samples stained with anti-CD151 primary antibody were incubated with a FITC-conjugated goat anti-mouse secondary antibody (Ancell Corporation).

Background fluorescence was established by negative controls and data were acquired using a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) collecting a minimum of 10,000 events. Analysis was performed using CellQuest software (BD Biosciences).

Adipogenic and osteogenic differentiation

Cells were plated at 3 x 10^3 cells/cm^2 and differentiated for 14 days in adipogenic medium using a repeated pulsed induction protocol and in osteogenic medium (de Girolamo 2009).

Lipid vacuoles were quantified by Oil Red O staining, unstained with isopropanol and absorbance was read at 490 nm (Perkin Elmer Victor X3 microplate reader; Perkin Elmer, Waltham, MA, USA).

Calcified matrix deposition was measured using Alizarin Red-S staining, unstained with cetylpyridinium chloride monohydrate (Sigma-Aldrich) and absorbance was read at 570 nm.

Chondrogenic differentiation in micromasses

4x10^5 cells were centrifuged (2 minutes at 232 *g*) and maintained for 28 days in chondrogenic differentiation medium (Colombini 2012). Additional 10 ng/ml of

BMP-6 was added to the ASCs (Peprotech, Rocky Hill, New Jersey, USA) (Estes 2006).

For histological analysis, pellets were fixed, embedded in paraffin, sectioned at 4 µm and stained with Alcian Blue (Sigma-Aldrich) to evaluate glycosaminoglycans (GAGs) deposition.

For GAGs quantification, pellets were digested (16 h, 60 °C) in PBE buffer containing L-cysteine (Sigma-Aldrich) and papain (Worthington). Samples were incubated with dimethylmethylene blue (Sigma-Aldrich) and absorbance was read at 500 nm.

Simulation of pro-inflammatory environment

Since IL1β is a cytokine well known to induce a pro-inflammatory environment in intervertebral disc and joint cartilage (Molinos M 2015; Le Maitre 2005; Le Maitre 2007a; Le Maitre 2007b; Kapoor 2011; Daheshia 2008; Johnson 2015), cells were stimulated by adding 1ng/ml of IL-1β to the culture medium for 48 h (Kim 2013). After this period, supernatant and cells were collected and used to assess the response to the treatment.

Gene expression analysis

Total RNA was isolated from cell lysates using the PureLink® RNA Mini Kit (Life Technologies) and quantified spectrophotometrically (NanoDrop, Thermo Scientific).

RNA were reverse-transcribed to cDNA employing the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA). Gene expression was evaluated by real time PCR (StepOne Plus, Life Technologies). cDNA was incubated with a PCR

mixture including TaqMan® Gene Expression Master Mix and TaqMan® Gene Expression Assays (Life Technologies).

The expression of the following stemness markers *NANOG*, Hs04260366_g1 and *POU5F1*, Hs04260367_gh was evaluated at P1 and P3. The expression of matrix metalloproteases *MMP1*, Hs00899658_m1, *MMP3*, Hs00968305_m1 and *MMP13*, Hs00233992_m1, of their inhibitors *TIMP1*, Hs00171558_m1 and *TIMP3*, Hs00165949_m1 and of growth factors *VEGFA*, Hs00900055_m1 and TGFB, Hs00998129_m1 was analyzed after IL1β treatment.

The previously validated *TBP*, Hs00427620_m1 was used as housekeeping gene (Lopa 2016). Data were expressed according to the ddCt method.

ELISA assays

The levels of IL-1, TNFα (Peprotech), IL-6 (R&D Minneapolis, MN, USA), IL-10 (), IL-1Ra, were evaluated in cell supernatant after 48h of IL1β treatment according to the manufacturer's procedures.

Quantification of nitrites

The amount of NO_2^- in the cell culture medium after 48h of IL1β treatment was determined by Griess method (Schmidt 1995). Culture medium was mixed with an equal volume of Griess reagent. After 15 minutes at room temperature, the optical density of the samples was measured at 540 nm.

Secretome preparation, concentration and cell treatments

ASCs culture medium was obtained from pooled confluent cells ($1,5x10^6$ cells) of two donors, after 24h of starvation. After centrifugation of 15ml

culture medium at 4000 xg for 30 min with Amicon Ultra-15 (Merck KGaA, Darmstadt, Germany), the obtained 2ml of concentrated secretome were used 5x for the cells treatment.

Statistical analysis

Data are expressed as mean ± standard deviation. Normal distribution of values was assessed by the Kolmogorov-Smirnov normality test. Statistical analysis was performed using Student's *t*-test for normally distributed data and Wilcoxon test in presence of a non-normal distribution (GraphPad Prism v5.00; GraphPad Software, San Diego, CA, USA). Level of significance was set at $p < 0.05$ (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). The number of data used for the statistical analyses is indicated in figure legends as "*n*", corresponds to independent experiments (Ranstam, 2012).

AUTHOR CONTRIBUTIONS

PDL: investigation; manuscript review.

MV: investigation; manuscript review.

LDG: supervision; manuscript review; funding.

CPO: investigation; manuscript review.

RC: patient recruitment, manuscript review.

LZ: patient recruitment, manuscript review.

MBB: patient recruitment, manuscript review.

CD: investigation; manuscript review; funding.

AC: conceptualization; project administration; investigation; formal analysis; paper writing; funding.

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3.3.1 MSCs Secretome: preliminary results from an *in vitro* model of cartilage cell inflammation

On the basis of the results described in Manuscript 3, the ability ASCs conditioned medium (CM) in reducing the catabolic response of articuarl chondrocytes (ACs) to IL-1β stimulation was tested. In the same experimental setting described above, ACs were stimulated with the inflammatory stimulus for 48 hours, in presence or absence of ASCs CM. The medium was concentrated to obtain an equivalent of 100.000 cells conditioning each ml. The inflammatory stimulus induced the expression of MMP-1 and VEGF in the ACs, while inhibiting TGFβ1 expression (Figure 1A). On the contrary, while in presence of ASCs CM, the expression of MMP-1 and VEGF was reduced, and an increase in TGFβ1 and TIMP-1 expression was observed (Figure 1B).

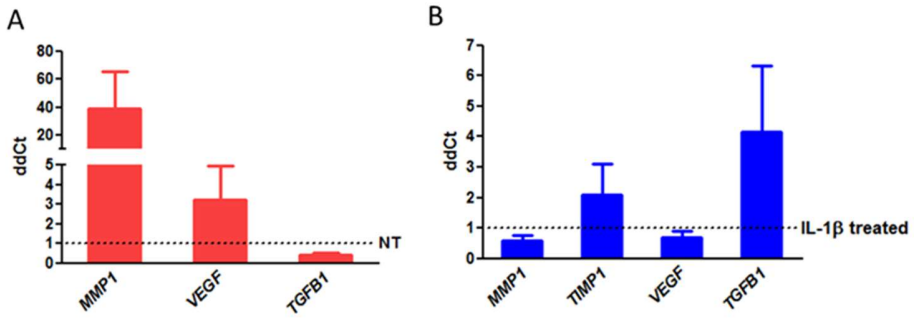

Figure 1. A) Expression of MMP-1, VEGF and TGFβ1 in ACs stimulated with IL-1β with respect to untreated (dotted line) (n=3). B) Expression of MMP-1, TIMP-1, VEGF and TGFβ1 in ACs treated with IL-1β and CM, with respect to IL-1β treated (dotted line) (n=3).

The investigation over the molecular mediators responsible for this effect is still ongoing, but a preliminary indication was obtained by TNFα and IL-1RA ELISA assay on ACs medium. The content of TNFα reduced in presence of CM, with respect to IL-1β stimulation alone, and at the same time, CM increased the content of IL-1RA (Figure 2). The alteration of the cell microenvironment by decreasing pro- and incrasing anti-inflammatory mediators represent a known ability of ASCs CM and is probably the main action behind the reduction of the catabolic markers in this experimental setting.

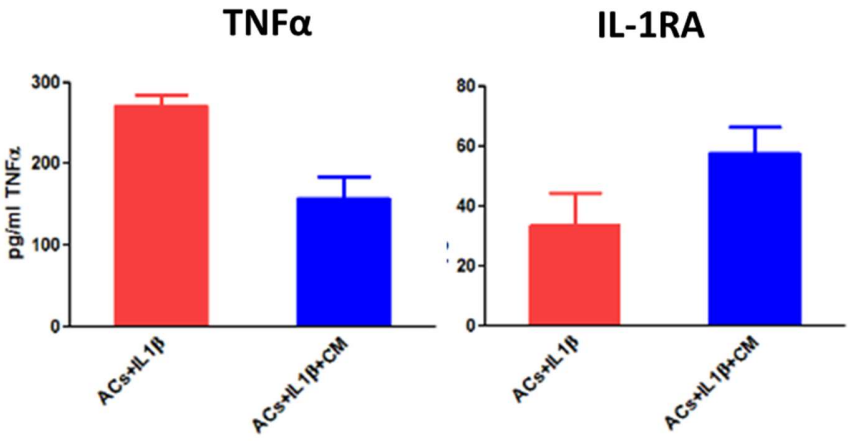

Figure 2. TNFα and IL-1RA concentrations in ACs medium after stimulation with IL-1β (red) or IL-1β and ASCs CM (blue).

4. Methods for the improvement of MSCs and progenitors stemness features and therapeutic potential (*Aim2*)

A second step in the progress of MSCs-based approaches is represented by the assessment of the best conditions for MSCs preparation, or "priming", to allow them to unleash their full potential.

In fact, the physiological ability of MSCs/progenitors cell in maintaining tissue homeostasis and promoting regeneration is often lost when cells are cultured *in vitro*. To overcome this issue, many different culture or selection conditions have been proposed. In particular, one of the main issue in developing feasible cell therapies is the choice of the most appropriate source of MSCs. Indeed, despite MSCs from different sources share many features, it has been recently demonstrated that there can be relevant differences among them. For this reason, in the setting of developing cell therapies for tendon-related conditions, we investigated different approaches for the enhancement of MSCs activity and yield with particular focus on TSPCs. At first, we investigated the influence of human basic fibroblast growth factor (bFGF) in the phenotype maintenance of

tendon cells and we explored the possibility to use clonal selection to enrich the tendon resident cell population with stem/progenitor cells *in vitro* (Manuscript 4: Viganò M et al., in publication in JEXO).

Other approaches to improve the homeostatic activity of MSCs are represented by the biophysical stimulations. As previously reported, the use of extracorporeal shock waves (ESWs) and pulsed electromagnetic fields (PEMFs) is a common practice in clinical orthopaedics for the treatment of different conditions, such as difficult bone fractures and cartilage lesions. Starting from these observations, we performed *in vitro* studies to assess the effect of these stimulations on tendon resident cells, and to evaluate the possible enhancement on their proliferative, trophic and anti-inflammatory activities, with the final aim to identify a potential rationale of ESWs and PEMFs in tendon-related pathologies too (Manuscript 5: de Girolamo et al., 2014; Manuscript 6: de Girolamo et al., 2015).

The modulation of MSCs characteristics would be of great importance for their application in tissue engineering. Indeed, a pre-treatment of MSCs to enhance their tissue specific abilities before transplantation would be of great help in avoiding phenotypic drift. Since there is no consensus in the current literature about a proper tenogenic induction protocol as well as the best MSCs source for cell-based therapy in tendon pathologies, we investigated the role of different biochemical factors in the induction of

tenogenic markers in TSPCs, ASCs and BMSCs (Manuscript 7: Viganò et al., in preparation).

4.1 Manuscript 4: Different culture conditions affect the growth of human tendon stem/progenitor cells (TSPCs) within a mixed tendon cells (TCs) population

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ABSTRACT

Background: Tendon resident cells (TCs) are a mixed population made of terminally differentiated tenocytes and tendon stem/progenitor cells (TSPCs). Since the enrichment of progenitors proportion could enhance the effectiveness of treatments based on these cell populations, the interest on the effect of culture conditions on the TSPCs is growing.

In this study the clonal selection and the culture in presence or absence of basic fibroblast growth factor (bFGF) were used to assess their influences on the stemness properties and phenotype specific features of tendon cells.

Results: The clonal selection allowed to isolate cells with a higher multi-differentiation potential, but at the same time a lower proliferation rate in comparison to the whole population. Moreover, the clones express a higher amounts of stemness marker *OCT4* and tendon specific transcription factor Scleraxis (*SCX*) mRNA, but a lower level of decorin (*DCN*). On the other hand, the number of cells obtained by clonal selection was extremely low and most of the clones were unable to reach a higher number of passages in cultures.

The presence of bFGF influences TCs morphology, enhance their proliferation rate and reduce their clonogenic ability. Interestingly, the expression of CD54, a known mesenchymal stem cell marker, is reduced in presence of bFGF at early passages. Nevertheless, bFGF does not affect the chondrogenic and osteogenic potential of TCs and the expression of tendon specific markers, while it was able to downregulate the *OCT4* expression.

Conclusion: This study showed that clonal selection enhance progenitors content in TCs populations, but the extremely low number of cells produced with this method could represent an insurmountable obstacle to its application in clinical approaches. We observed that the addition of bFGF to the culture medium promotes the maintenance of a higher number of differentiated cells, reducing the proportion of progenitors within the whole population. Overall our findings demonstrated the importance of the use of specific culture protocols to obtain tendon cells for possible clinical applications.

Keywords: FGF, tendon, tendon cells, clonal selection, progenitor cells, tenocytes

INTRODUCTION

Tendon cells (TCs) represent 5% of tendon tissue weight and are the main responsible for the maintenance of tissue homeostasis. The majority of these cells (90-95%) consists in a mixed population made of terminally differentiated tenocytes, representing the predominant cell type, and tendon stem/progenitor cells (TSPCs), which show several features of stem cells (Bi Y et al 2007; Lui PP et al 2011); the remaining 5-10% is represented by chondrocytes, synoviocytes and vascular endothelial cells (Kannus P et al, 2000). The possibility to use TCs, and specifically TSPCs, in regenerative medicine approaches is currently under investigation, with promising preliminary results expecially deriving from pre-clinical models. Their application ranges from injective treatment (Ni M et al 2012; Chen L et al 2012) to the use of cell-seeded scaffolds as a form of surgical augmentation (Cao Y et al 2002; Chen JM et al 2007; Stoll C et al 2011; Chen J et al 2011). To date, due to the absence of specific cell markers suitable for the

sorting of tendinous subpopulations containing cells at different stages of differentiation, culturing tendon cells at low density represents the most used method to isolate specific progenitor subpopulations.. Although their real effectiveness is still to be clearly demonstrated, several studies have reported satisfactory results using both low density culture and single colony harvesting to isolate TSPCs from a mixed tendon cell population in rat and rabbit (Lui PP et al 2011, Zhang P et al 2010, Bi Y et al 2007, Rui YF et al 2010). Nevertheless, how the culture conditions can influence the TCs population features and its enrichment in progenitor content is still debated, at least for what concern human-derived cells. Different growth factors have been also used in order to enhance or reduce the proportion of either terminally differentiated tenocytes or tendon stem/progenitor cells within TCs culture. TGF-β, IGF-1, BMPs and PDGF were found to be able to induce tenocyte proliferation and, at the same time, to enhance the expression of tendon markers (Gaspar D et al 2015). In addition, IGF-1 could enhance the stem properties of TSPCs in culture (Hollyday C et al 2013), whereas basic fibroblast growth factor (bFGF) is known to enhance proliferation of fibroblasts (Yun YR et al 2010) and to maintain tendon marker expression in cultured tenocytes as well as to enhance their proliferation in combination with PDGF or IGF-1 (Caliari SR et al 2013; Qiu Y et al 2013; Costa MA et al 2006).

In this study we have compared different culture conditions of human TCs, assessing their possible influences on the stemness properties and phenotype specific features of tendon cells. In particular, we focused on the use of bFGF and clonal isolation, with the aim to investigate if these approaches will allow to

increase the number of tendon progenitors having a translational potential for regenerative medicine applications.

MATERIALS & METHODS

Tendon cells (TCs) isolation, culture conditions and clonal selection

All of the procedures were carried out at Galeazzi Orthopedic Institute (Italy) with Institutional Review Board approval (M-SPER-014.ver7 for the use of surgical waste). The donors gave their written informed consent to the participation to the study. Gracilis and semitendinous tendons were harvested from leftover tissue that would otherwise be discarded of 8 donors (6 males, 2 females; mean age 31.1±10.9 years) who underwent anterior cruciate ligament reconstruction. Tendon cells were isolated from fragments by enzymatic digestion (24 hours, 37°C,) with 0.3% w/v type I collagenase (Worthington Biochemical Corp., NJ, USA) in high glucose DMEM (HG-DMEM, Life Technologies, Carlsbad, CA, USA) (Rui Y et al 2010; de Girolamo L et al 2013). After the digestion, the samples were filtered through a 100 µm cell strainer (Becton, Dickinson and Co., NJ, USA) and centrifuged (300 g, 5 minutes). The cells were counted and plated at a density of $5x10^3$ cells/cm^2 in complete medium (CM) consisting of HG-DMEM supplemented with 10% FBS (Sigma-Aldrich, St. Louis, MO, USA), 50 U/mL penicillin, 50 mg/mL streptomycin, 2 mM L-glutamine (all from Life Technologies), and maintained in incubator at 37°C in a humidified atmosphere with 5% CO_2. Once they reached 80%–90% of confluence, the cells were detached with 0.5% trypsin/0.2% EDTA (Sigma-Aldrich) and plated at a density of $3x10^3$ cells/cm^2 and cultured in CM in absence (TCs-) or in presence (TCs+) of 5 ng/mL bFGF (Peprotech, NJ, USA). The cells were used for the

experiments until passage 4 (P4). A clonal selection was also performed on all the 8 cell populations included in this study. Cells at P1 were plated at low density, 50 cells/cm^2 (Rui Y et al 2010), and cultured in CM without bFGF. After 3 weeks of culture, the colonies were detached by 0.5% trypsin/0.2% EDTA using Pyrex® cloning cylinder (Corning, NY, USA). Clones were further expanded at normal density to P4 and the multi-differentiation potential and the gene expression of each colony were investigated. Cells cultured at normal density ($5x10^3$ cells/cm^2) were used as controls.

Morphological evaluation

TCs+ and TCs- were daily observed at the optical microscope and their morphology at P4 was evaluated: cells were fixed in 4% paraformaldehyde solution, nuclei were stained with DAPI (1 µg/ml, Life Technologies) and F-actin filaments were stained with Phalloidin (6.6 M, Life Technologies). The samples were then imaged through a fluorescence microscope (Olympus IX71).

Doubling time evaluation

The doubling time (DT) of both TCs+ and TCs- was recorded from P2 to P4 and calculated according to the following formula: DT = CT/ln(Nf-Ni)/ln2, where CT is the cell culture time (hours), Nf is the final number of cells and Ni is the initial number of cells (Staszkiewicz J et al 2008).

Clonogenic ability assay

A colony-forming unit-fibroblast (CFU-F) assay was performed at P2 and P4. TCs + and TCs - were plated in 6 well plate at different seeding density (1 cell/cm^2; 3 cells/cm^2; 6 cells/cm^2; 12 cells/cm^2; 24 cells/cm^2; 48 cells/cm^2) and cultured in CM with 20% of FBS (Lopa S et al 2014). After 14 days, cells were fixed with 4% paraformaldehyde, stained with 2.3% Crystal Violet staining (Sigma-Aldrich) for

10 min at room temperature and then counted. CFU-F frequency was established by scoring the individual colonies composed of at least 30 cells and expressed as a percentage relative to the number of seeded cells.

Flow cytometry

The immunophenotype of TCs+ and TCs- at P2 and P4 was evaluated by Fluorescence-Activated Cell Scanning (FACS) analysis. Cells were washed twice in cold FACS buffer (phosphate-buffered saline without Ca/Mg^{2+}, 2% fetal bovine serum and 0.1% NaN_3). For each sample, 2.5×10^5 cells were single-stained with the following anti-human primary monoclonal antibodies: fluorescein isothiocyanate-conjugated CD90, CD13 and CD45 (Ancell Corp., MN, USA)); biotinylated-conjugated CD34, CD54 and CD105 (Ancell Corp.); and phycoerythrin-conjugated CD73 (Miltenyi Biotec, Germany). Streptavidin–phycoerythrin (Ancell Corp.) was used to reveal the expression. Background fluorescence was set up by negative controls and data (10,000 cell fluorescence events) were acquired using a FACSCalibur™ flow cytometer (BD Bioscences, NJ, USA) and analyzed by CellQuest™ software (BD Bioscences).

RNA extraction and Real Time PCR

Gene expression of TCs+, TCs- and clones at P2 and P4 was evaluated by real time PCR (StepOne Plus, Life Technologies). Total RNA was extracted by PureLink® RNA Mini Kit (Life Technologies) and reverse transcripted to cDNA (5 min at 25°C, 30 min at 42°C and 5 min at 85°C) using an iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, CA, USA). Twenty ng of cDNA were used as template and were incubated with a PCR mix (50°C for 2 min, 95°C for 10 min, 40 cycles at 95°C for 15 s and 60°C for 1 min) containing TaqMan® Universal PCR Master Mix and Assays-on-Demand Gene expression probes (Life Technologies) for the following

genes: *KLF4* (Hs00358836_m1), *OCT4* (Hs04260367_gh), *MKX* (Hs00543190_m1), *DCN* (Hs00370385_m1) and *SCX* (Hs03054634_g1). Reactions were performed with Applied Biosystems® StepOnePlus™ (Life Technologies). The fold change in expression was normalized against the expression of the housekeeping gene *GAPDH* (Hs99999905_m1). Two replicates were analyzed for each experimental group. Data were expressed according to the ddCt method.

Multi-differentiative potential

Adipogenic potential

TCs+, TCs- and clones at P4 were seeded in 24-well plates at 10^4 cells/cm^2 and differentiated for 14 days in a pulsed adipogenic medium (de Girolamo L et al 2009) 3 days of induction in CM supplemented with 1 µM dexamethasone, 10 µg/mL insulin, 500 µM 3-isobutyl-1-methylxanthine and 200 µM indomethacin (all from Sigma-Aldrich), followed by 3 days of maintenance in CM supplemented with 10 µg/mL insulin. The cells were fixed in 10% neutral buffered formalin for 1 h and stained with Oil Red O (Sigma-Aldrich) for 15 min to evaluate lipid vacuoles formation. Oil Red O was unstained with 100% isopropanol and then quantified by absorbance at 490 nm (Perkin Elmer Victor X3 microplate reader; Perkin Elmer, Waltham, MA, USA).

Osteogenic potential

TCs+, TCs- and clones at P4 were seeded at 10^4 cells/cm^2 and differentiated for 14 days in osteogenic medium consisting of CM supplemented with 10 nM dexamethasone, 10 mM glycerol-2-phosphate, 150 µM L-ascorbic acid-2-phosphate and 10 nM cholecalciferol (all from Sigma- Aldrich) (de Girolamo L et al 2009). The extracellular calcified matrix deposition was measured using Alizarin Red-S staining. Cells were fixed with ice-cold 70% ethanol for 1 h and

stained with 40 mM Alizarin Red S (pH 4.1; Fluka-Sigma Aldrich, MO, USA) for 15 min. The dye was extracted with 10% cetylpyridinium chloride monohydrate (Sigma-Aldrich) in 0.1 M phosphate buffer (pH 7.0) and the absorbance was read at 550 nm (Perkin Elmer Victor X3 microplate reader).

Chondrogenic potential

TCs+, TCs- and clones at P4 were seeded at 10^4 cells/cm^2 and cultured for 21 days in chondrogenic medium consisting of HG-DMEM supplemented with 1% of FBS, 2 mM L-glutamine, 50 U/mL penicillin, 50 mg/mL streptomycin, 1 mM sodium pyruvate (all from Sigma-Aldrich), 1% ITS+1 (1.0 mg/mL insulin from bovine pancreas, 0.55 mg/mL human transferrin, 0.5 µg/ mL sodium selenite, 50 mg/mL bovine serum albumin and 470 µg/mL linoleic acid, Sigma-Aldrich), 0.1 mM dexamethasone, 0.1 mM L-ascorbic acid-2-phosphate, 10 ng/ml TGF-β1 (Peprotech) (Barbero A et al 2004). The cells were fixed with 10% neutral buffered formalin solution, rinsed with distilled water, and stained with Alcian Blue solution (pH 2.5) for 30 minutes (Sigma-Aldrich) to evaluate glycosaminoglycan deposition. The dye was extracted with guanidine hydrochloride (6M) and the absorbance read at 650nm (Perkin Elmer Victor X3 microplate reader) (Ruzzini L et al 2014).

Statistical analysis

Data are expressed as means ± standard deviations. GraphPad Prism v5.0 software (GraphPad Software Inc., La Jolla, CA, USA) was used to perform all the analyses. To assess for adjustment of series of values to normal distribution, the Kolmogorov-Smirnov test was applied. Student's t test was applied to compare values between groups when data were normally distributed, otherwise Mann-

Whitney's test was performed. p values <0.05 were considered as statistically significant. (*p < 0.05).

RESULTS

Clones obtained by TCs seeded at low density show a higher multi-differentiation potential and stemness marker expression, but a lower proliferation rate, in comparison to the whole population

Among the 8 TC populations seeded at low density (50 cell/cm^2), only three gave rise to clones and allowed to proceed with the further experiments. Due to the low number of samples, no statistical analysis was performed, and all following observations refers to trends.

From each of these, a range of 3-11 clones were isolated, with 6.3±4.9x10^3 cells per clone at P2. At P3 the number of cells increased to 4.8±4.2x10^4, even if only the 72±27% of clones survived. At P4, this proportion decreased to 21±29%, with a mean cell count of 1.1±0.6x10^4.

The osteo-differentiated clones showed an increase of +36% in matrix deposition with respect to whole population. Similarly, an increase in their chondrogenic ability was observed in chondro-differentiated clones with respect to whole population (+102%). Indeed, each clone demonstrated extremely different features in terms of differentiation potential, ranging from great to none ability to produce calcified matrix or glycosaminoglycans.

Just one population over the 8 tested allowed a suitable cell harvesting for mRNA extraction. We obtained 6 different clones from this population, and they showed a quite variable relative expression in all analyzed markers, with no particular correlation with their differentiation ability. Interestingly, the mean

relative expression of *OCT4* and *SCX* in the clones resulted 6-fold higher than the whole population, while a 10-fold decrease was observed for what concern *DCN*. Moreover, a positive correlation was found between the expression of *OCT4* and *SCX* among clones of the same population (Pearson's R^2=0.89, $p<0.05$), while a negative correlation was observed comparing *SCX* and *KLF4* (Pearson's R^2=-0.91, $p<0.05$).

The presence of bFGF influences proliferation rate, clonogenic ability, the expression of CD54 and OCT4

TCs cultured in presence or absence of bFGF reveal similar morphology, even if a more pronounced fibroblast-like morphology was observed when cultured in presence of bFGF (Fig. 1A). As expected, the proliferation rate was higher in presence of bFGF, with a doubling time of 70±26 and 62±16 hours at P3 and P4, respectively (-18% and -31% vs TCs-, $p<0.05$) (Fig. 1B). On the other hand, TCs- showed a higher clonogenic ability both at P2 and P4

3.2%±2.3% and 4.2%±1.6%, respectively) in comparison with TCs+ (0%±0.0%and 1.6%±1.1%) (Fig. 1C, D).

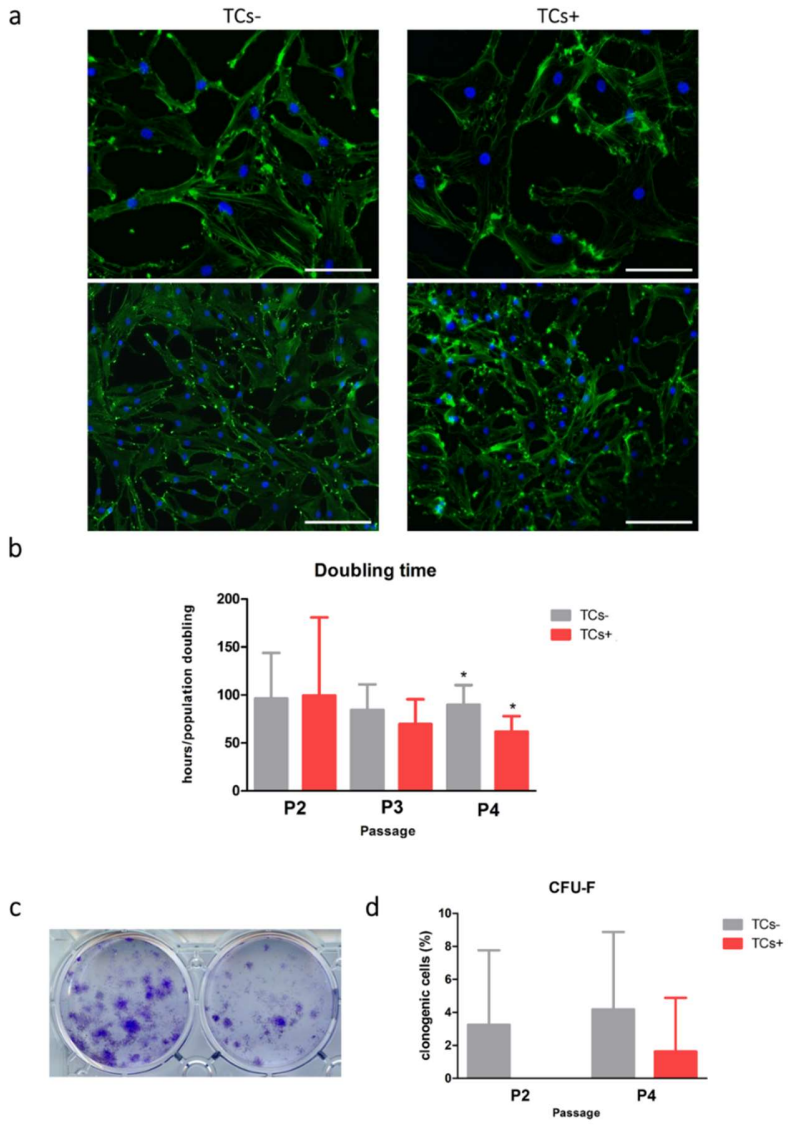

Figure 1. Morphology and clonogenic potential. *(a) Cells in culture at P4 and after DAPI/phalloidin staining (scale bars 50 µm for upper panel and 100 µm for lower panel). (b) Proliferation ability during passages in culture expressed as*

*mean of doubling times. Levels of significance: *p<0.05 (c) Representative micrographs of CFU-F at P4. (d) Percentage of clonogenic cells at P2 and P4 (n=4).*

Cell surface marker analysis was performed during expansion (P2 and P4). The presence of bFGF did not influence the percentage of cells positive for the stemness-related markers CD13, CD73, CD90, as well as the percentage of cells negative for CD34, CD45 expression, which are known to be hematopoietic cell markers. The only difference between TC+ and TC- was observed in CD54 expression, which was significantly higher expressed in TCs- at both P2 and P4 in comparison with TCs+ (p<0.5). For both the cell types, an increasing trend of CD54 expression from P2 to P4 was observed. The complete data concerning cell surface marker expression are shown in Table 1.

Table 1. Cell surface markers expression.

	Surface Markers	CD13	CD34	CD45	CD54	CD73	CD90	CD105§
TCs-	P2	99.7%± 0.1%	3.7%± 1.9%	2.6%± 1.6%	85.7%± 6.5%*	99.8%± 0.1%	99.1%± 0.7%	98.3%± 1.1%
	P4	99.9%± 0.1%	1.7%± 0.7%	0.8%± 0.7%	92.1%± 3.0%*	99.9%± 0.1%	96.8%± 0.5%	98.4%± 0.5%§

TCs+	**P2**	99.7%± 0.1%	2.9%± 3.2%	0.8%± 0.7%	46.6%± 29.2%	99.7%± 0.4%	99.0%± 0.5%	98.2%± 0.7%
	P4	99.7%± 0.4%	2.7%± 1.5%	0.4%± 0.3%	82.5%± 9.6%	99.8%± 0.4%	98.6%± 0.2%	98.6%± 0.3%§

*Percentage of positive TCs- and TCs+ for the whole panel of surface markers tested at P2 and P4 (n=4). * p<0.05 TCs- vs TCs+.*
§ Data at passage 5.

Both TCs+ and TCs- cultured for 14 days in adipogenic medium showed no appreciable intracellular lipid vacuole production in comparison with cells maintained in non-inductive medium (data not shown).

Both in presence or absence of bFGF, TCs were able to differentiate towards the osteogenic lineage. Indeed, after 14 days of culture in osteogenic medium the deposition of calcified matrix was significantly higher in differentiated cells with regards to controls (3.9 fold in TCs- and 3.0 fold in TCs+ $p<0.05$, Fig. 2A,B), with no differences between culture conditions. At the same manner, the chondrogenic potential measured after 21 days of culture in chondro-inductive condition, was similar between the two populations with significantly higher amount of glycosaminoglycans in comparison with the respective controls (2.9 fold in TCs- and 2.6 fold in TCs+, $p<0.05$, Fig. 2C, D). Interestingly, the basal level of GAG and calcified matrix deposition does not differs between the two populations, also in non-inductive conditions.

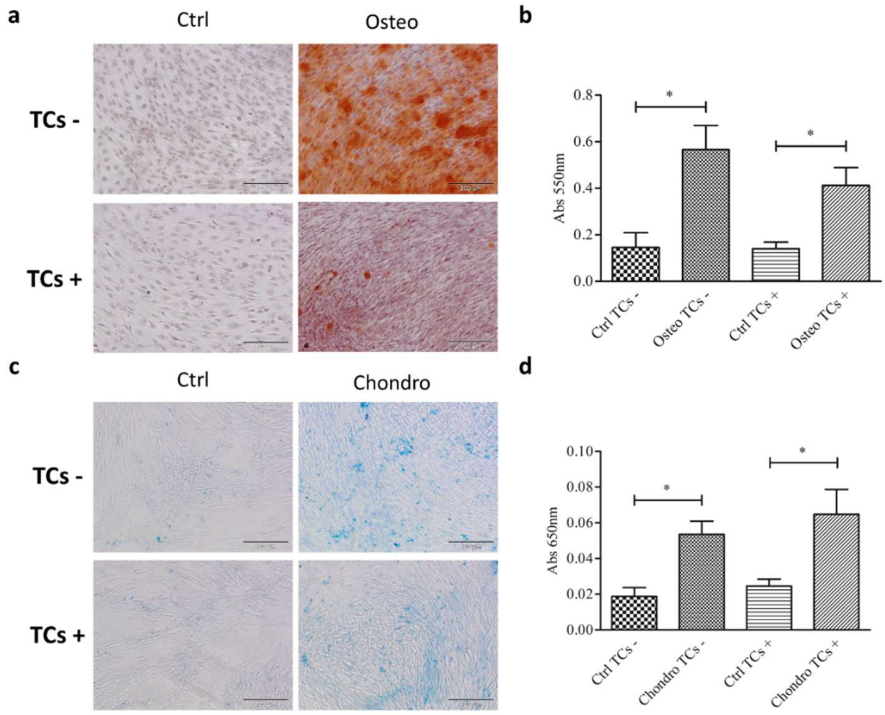

Figure 2. Multi-differentiative potential. *(a) Osteogenic differentiated TCs- and TCs+ in culture and after AR-S (scale bars 200 µm). (b) Quantification of calcified matrix by AR-S staining and extraction in undifferentiated (Ctrl) and osteogenic-differentiated (Osteo) cells (n=6). (c) Chondrogenic differentiation of TCs- and TCs+ in culture and after Alcian Blue staining (scale bars 500 µm). (d) Quantification of glycosaminoglycans deposition by Alcian Blue staining and extraction in undifferentiated (Ctrl) and chondrogenic-differentiated (Chondro) cells (n=4). Levels of significance: * p<0.05.*

A significant decrease of 1.36 fold ($p < 0.05$) in the expression of *OCT4*, was observed in TCs+ in comparison with TCs- cultured for 4 passages. Moreover, the cells cultured in presence of bFGF showed a clear decrease in *OCT4* expression from P2 to P4, even if this difference was not statistically significant. On the contrary, *KLF4* expression was stable in both the cell culture conditions and during passages (Fig. 3).

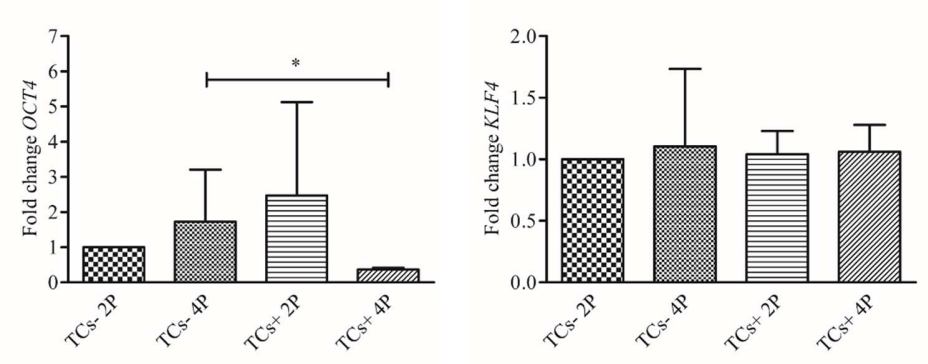

***Figure 3. Gene expression of stemness markers**. OCT4 and KLF4 genes expression in TCs- and TCs+ at P2 and P4 normalized to GAPDH (n=6). Levels of significance: * p<0.05.*

The expression of *MKW*, *DCN* and *SCX* was measured at P2 and P4. Slight and no statistically significant differences were observed between TCs- and TCs+. In particular the TCs- expression of *DCN* increased with time in culture, and the presence of bFGF further up-regulated its expression (Fig. 4).

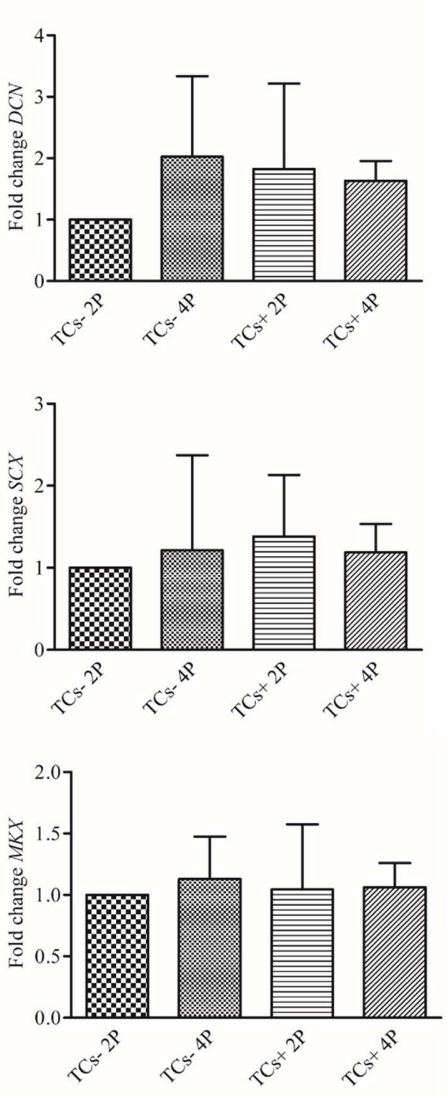

Figure 4. Gene expression of tendon markers*. MKX, DCN and SCX genes expression in TCs- and TCs+ at P2 and P4 normalized to GAPDH (n=6).*

DISCUSSION

Adult mesenchymal stem cells represent a tool for clinical applications in regenerative medicine (Ménard C et al 2013). In this field, the use of MSCs for the treatment of tendon disorders is still far from the clinical practice due to the lack of knowledge about the tenogenic potential of the MSCs. Therefore, since the description of tendon stem/progenitor cells in 2007 by Bi and colleagues (Bi Y et al 2007), the possible use of autologous tendon cells in tendon regenerative medicine approaches is subject of a growing interest (Ho JO et al 2014). Nevertheless, the presence of different subpopulations among the tendon resident cells represents a largely unexplored field of investigation. The lack of a specific terminology and the difficulties in purifying, expanding and maintaining the different cellular subsets are the main obstacles in this field (Docheva D et al 2015). In view of future applications of regenerative approaches for the treatment of tendon disorders, it would be crucial to define the most suitable culture conditions to isolate the different sub-populations within tendon cells, and to improve their ability in promoting tendon healing and regeneration. For this purpose, different culture conditions have been proposed to enrich *in vitro* cultures with one population or the other, as the application of specific growth factors or patterned substrates, such as tendon derived matrix [Zhang J et al 2011] to simulate the features of the native microenvironment of tenocytes [Gaspar 2014].

In our study we investigated the presence of Tendon Stem/Progenitor Cells (TSPCs) within the tendon resident cells (TCs) isolated from human gracilis and semitendinosus tendons, and we assessed the effects of bFGF and clonal selection as possible strategies to modulate their predominance in culture.

Almost 25% of the TC populations analyzed gave rise to few clones when cultured in clonal selection conditions. Indeed, the low content of progenitor cells in tendons is a well known limitation to their application [Bi Y et al., 2007]. The clones showed a higher mean ability to differentiate toward osteogenic and chondrogenic lineages, as revealed by the production of calcified matrix and glycosaminoglycans, respectively, in comparison with the corresponding whole population, with high differences in the performance of each clone though. Interestingly, the stemness marker *OCT4*, as well as the early tenogenic marker *SCX*, were up-regulated in clones with regards to the whole population, and the expression of these two genes was positively correlated. On the contrary, the late tenogenic marker *DCN* was less expressed in clones in comparison with whole population. Moreover, the expression of the stemness marker *KLF4* resulted slightly higher in clones with respect to the cells cultured at normal density, in particular in presence of bFGF. Nevertheless, the *KLF4* pattern of expression could vary between different conditions, such as stages of cell differentiation, and this could explain the negative correlation between this marker and *SCX* (Zhang P et al 2010).

As expected, all these data showed that the selected clones exhibited a more undifferentiated phenotype in comparison with whole TC population. Despite these encouraging data, the low number of cells that can be isolated by clonal selection make this approach hardly applicable to cell-based therapy, until the identification of growth factors able to enhance the cell yield from isolated clones. The use of bFGF would help reaching this goal, but since it resulted inefficient for the stemness maintenance, we would not recommend it for this purpose. Indeed, bFGF is often used to maintain the cell multipotency in many

cells types, and, specifically in tendon cell populations, to enhance the tendon lineage differentiation (Tsutsumi S et al 2001; Hankemeier S et al 2005; Tokunaga T et al 2015). However, its role in promoting the predominance of tendon progenitor cells within the whole tendon cell population have not been investigated so far.

This growth factor, known to enhance cell proliferation in many cell types (Ornitz DM et al 2015), exerted the same effect on TCs. The evaluation of the immunophenotype of TCs treated or not with bFGF, showed no difference in term of CD13, CD73, CD90, CD34 and CD45 expression. On the contrary, starting from early passages until passage 4, the expression of the adhesion molecule CD54 (Intercellular Adhesion Molecule-1, ICAM-1) was higher in TCs cultured in absence of bFGF in comparison with TCs+. CD54 has been characterized as one of the peculiar mesenchymal stem cell markers (Calloni Ret al 2013) and it is important in inhibiting the osteogenic differentiation of mesenchymal stem cells (Xu FF et al 2014). The higher expression of CD54 in TCs- in comparison with TCs+ and the increase from passage 2 to passage 4 of its expression in both the populations could indicate a progressive enrichment of progenitors in tendon cells during culture, particularly when maintained in absence of bFGF.

Accordingly, for what concern stemness features, TCs- showed a higher clonogenic ability and *OCT4* expression in comparison with TCs+. Since this gene is known to have a role in sustaining self-renewal capacity of adult stem cells (Niwa H et al 2007), the higher expression in TCs- even at the latest passage (P4) in comparison with TCs+, suggest that the lack of bFGF allowed for the maintenance of a more undifferentiated cell phenotype. The assessment of the

multilineage differentiation of TCs in term of adipo, chondro- and osteogenic potential confirmed the findings of a previous study (Stanco D et al 2015).
Indeed, our study showed that both TC- and TCs+ had no appreciable adipogenic potential, but they were able to deposit a consistent amount of calcified matrix and glycosaminoglycans in comparison with control cells, with no appreciable differences between them. Similarly, the expression of tendon markers such as *MKW*, *DCN* and *SCX* was not influenced by the presence of bFGF.
A limitation of the present work is represented by the low number of cells obtained by clonal selection, resulting in restrictions to the possible analysis. Moreover, the lack of a specific marker of tendon progenitor would not allow the application of cell sorting for the enrichment of the progenitor population. Further studies to investigate this aspect will be crucial for the development of the cell-based therapies for tendon regeneration.

CONCLUSION

In conclusion, our study highlights the importance of modulating different culture protocols to obtain useful tendon cells for clinical application. In particular, the addition of bFGF to the culture media caused a loss of stemness features such as clonogenic ability and OCT4 expression, without causing the loss of the tenogenic phenotype. On the other hand, in order to obtain a greater amount of tendon progenitor cells the use of bFGF is not suggested.
Taken together our results showed how much the different cell populations within the tendon tissue are sensitive to the biochemical environment. Further *in vitro* as well as pre-clinical studies are needed to better correlate the use of different stimuli to the cell responsiveness. Moreover, since tendons are known

to be greatly affected to mechanical stress and forces, it would be interesting to identify a possible synergistic effect of biochemical with physical factors.

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4.2 Manuscript 5: Soft-focused extracorporeal shock waves increase the expression of tendon specific markers and the release of anti-inflammatory cytokines in an adherent culture model of primary human tendon cells

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ABSTRACT

Focused extracorporeal shock waves (ESW) have been shown to up-regulate the expression of collagen and to initiate cell proliferation in healthy tenocytes and to positively affect the metabolism of the tendons, promoting the healing process. Recently, soft-focused extracorporeal shock waves have been also shown to have a significant effect on tissue regeneration. However, very few *in vitro* reports deal with the application of this type of shock waves on cells and, in particular, no previous studies investigate the response of tendon cells (TCs) to this impulse. We devised an original model to investigate the *in vitro* effects of soft-focused shock waves on a human heterogeneous population of resident tendon cells in adherent monolayer culture. Our results showed that soft-focused ESW treatment (0.17 mJ/mm^2) was able to induce a positive modulation of cell viability, proliferation, tendon-specific marker expression, as well as the release of anti-inflammatory cytokines. This could prefigure a new rationale for employing routinely soft-focused shock waves to treat the failed healing status which distinguishes tendinopathies.

KEYWORDS

Soft-focused extracorporeal shock waves; tendon cells; tendinopathies; cell proliferation; transforming growth factor β; vascular endothelial growth factor; cytokines; tendon-specific markers.

INTRODUCTION

Tendinopathies are common pathologies, particularly among sportspeople, and represent about 45% of all musculoskeletal injuries (Maffulli and Kader 2002).

Aim 2

Despite the progress in the treatment of tendinopathies, several aspects related to the complex tendon pathophysiology remain unclear (Del Buono et al. 2011). There is still debate regarding the true role of inflammatory insult and of overload in the activation of the processes which gradually produce degenerative changes of the tendon structure as a result of qualitative and quantitative alteration of tenocytes (Abate et al. 2009; Fredberg and Stengaard-Pedersen 2008; Cook and Purdman 2009).

As recently shown, along with tenocytes (up to 90-95%) and a limited number of chondrocytes and endothelial cells, human tendons are also composed of tendon stem/progenitor cells (TSPCs) that have universal stem cell characteristics such as clonogenicity, multipotency and self-renewal capacity (Bi et al. 2007). They also help maintain the homeostasis of the tendon (Bi et al. 2007).

In the past decades several conventional conservative approaches to treat tendinopathies have been evaluated (Andreas and Murrell 2008), including extracorporeal shock waves (ESW) (Notarnicola et al. 2012). Shock waves used in medical practice are non linear, single, sonic pulses with a broad frequency spectrum ranging from 16 Hz to 20 MHz. They are characterized by a rapid (<10nsec) and short (<10µsec) fluctuations of the positive acoustic energy (up to 10-100 MPa) followed by a low tensile phase, near to 10%-20% of the positive pressure peak (Ogden et al 2001). These phenomena act on living tissues through specific pathways of mechanotransduction, affecting cell membrane polarization, triggering free radicals formation and modulating gene expression and growth factors production (Wang et al. 2002). In healthy tenocytes shock waves have been shown to up-regulate the expression of collagen and to initiate

cell proliferation (Vetrano et al. 2011), whilst in human tenocytes derived from pathological tendons, a decrease of collagen type I and scleraxis has been observed (Leone et al. 2012). In addition, different animal models have demonstrated that ESW affect local blood flow and metabolism of the tendon, and promote the healing process by increasing the expression of typical growth factors (TGFβ1, IGF-I) (Chen et al. 2004), as well as the synthesis and the organization of the collagen fibers (Ohran et al. 2004). Moreover, one of the most interesting aspects of the effect of shock waves on tendons is that the acoustic impulse lowers the expression of matrix metalloproteases (MMPs) and pro-inflammatory interleukins (IL) (Han et al. 2009), which are known to have a role in the pathogenesis of tendinopathies.

The dose-related response of the cells to shock waves seems to be closely related to the type of the generator as well as to different energy settings (Martini et al. 2006). *In vivo* experiments on rabbit Achilles tendon showed histopathological changes which varied from an inflammatory peritendinous reaction at lower energy flux density (EFD) values, to capillary disruption, erythrocyte extravasations, necrosis of the tendon fibers and fibroblast proliferation for higher levels of EFD (Rompe et al. 1998). Again, in a model of rat tenocytes, the best results in terms of proliferation and collagen synthesis, were observed at lower values of EFD and number of shocks, together with an immediate and transient increase of the mediator nitric oxide (NO) (Chao et al. 2008).

Another variable that can influence the outcome of a shock wave treatment is given by the characteristics of the focus. Recently, soft-focused shock waves have been shown to have a significant effect on tissue regeneration (Kuo et al. 2009). The peculiarity of soft-focused shock waves lies in the possibility to deliver

the energy to a larger area while the temporal feature of the impulse remain unvaried. Thanks to this feature, soft-focused shock waves are especially suitable for *in vitro* experiments, particularly when cells adhere to the culture plate.

On the basis of these observations, we devised an original model to investigate the *in vitro* effects of soft-focused shock waves on a human heterogeneous population of resident tendon cells (TCs) in adherent monolayer culture. For our purpose, we used an electro-hydraulic device in which the shock waves are produced by the high voltage discharge of an electrode placed in the water-containing compartment. The primary shock waves front is convoyed by a parabolic reflector in a second, almost parallel, large ovoid shaped focus (soft-focus) (Mittermayr et al. 2011). The probe (OP 155 applicator) generating shock waves was coupled with a patented waterbath for the treatment of cell cultures. After treatments, TCs viability, DNA content, specific tenogenic gene expression, anti- and pro-inflammatory cytokine release and nitric oxide production were evaluated. All the experiments were performed on seven different cell populations, isolated from small portions of healthy semitendinosus and gracilis tendons of seven patients who had undergone anterior cruciate ligament (ACL) reconstruction. To our knowledge, this is the first experiment of its kind to be reported, also including the description of the soft-focused SW effects on cytokine release.

MATERIALS AND METHODS

TC isolation and culture expansion

All the procedures were carried out with Institutional Review Board approval. Discarded fragments of semitendinosus and gracilis tendons were collected from

7 healthy young donors (mean age 29±7 years) who underwent anterior cruciate ligament (ACL) reconstruction with autologous hamstrings at our hospital. All patients had given written consent to the procedure. To isolate tendon cells (TCs), the tendon tissue was minced and digested enzymatically with 0.3% type I collagenase (Worthington, Lakewood, NJ, USA) in DMEM (Sigma-Aldrich, St. Louis, MO, USA) with continuous agitation for 15 hours at 37°C. The isolated nucleated cells were then cultured at $5x10^3$ cells/cm^2 in complete medium consisting of DMEM high glucose, 10% fetal bovine serum (FBS; Sigma-Aldrich), 50 U/ml Penicillin, 50 µg/ml Streptomycin, 2 mM L-glutamine (all from Life Technologies, Carlsbad, California, USA) and supplemented with 5 ng/ml basic fibroblast growth factor (b-FGF; Peprotech, NJ, USA). Cell cultures were maintained at 37°C in humidified atmosphere with 5% CO_2, changing culture medium every 3 days. The TCs remained quiescent for about 5 days before starting to proliferate rapidly. When TCs reached 80–90% of confluence, they were detached by incubation with 0.5% trypsin/0.2% EDTA (Sigma-Aldrich) and then expanded at a density of $3x10^3$ cells/cm^2. Cells from passages 2 to 4 (P2–P4) were used for the experiments.

***In vitro* extracorporeal shock wave treatment (ESWT)**

In order to allow a complete treatment of the entire cell culture, $4x10^5$ TCs were plated onto a limited area of the T25 flask that could be reached by the shock wave impulses. For this purpose, the culture flasks were kept in an inclined position during culture for three days before treatment. Immediately before treatment with ESW, the culture flasks were completely filled with DMEM to avoid air bubbles blocking the shock wave impulses, and then then they were immersed in a patented system for ESW cell treatment (Holfeld et al. 2009). This

system consisted of a Plexiglas box containing degassed water to avoid cavitation phenomena, with a heater plate to avoid thermic shock to the cells during treatment (water at 37°C). On the anterior wall, the box had a rounded porthole covered by a silicone membrane on which the shock wave applicator was applied. Standard ultrasound transmission gel was used to convey the acoustic energy to the submerged flask containing the cells. To hold the cell culture flask in place a slotted bar, a flask holder and a locking screw were used to allow the flask to be positioned at the selected distance from the shock wave source. Finally, on the back wall, a wedge shaped absorber was used to avoid shock waves being reflected and causing disturbance.

The electro-hydraulic shock wave device was equipped with an soft-focused applicator (OP155-Orthogold 100, MTS Europe GMBH Konstanz–Germany). On the basis of preliminary tests to ascertain the optimal EFD in term of cell viability and proliferation (data not shown) and on the basis of the energy flux we use in the clinical practice to treat tendinopathies, the cells were treated with an EFD of 0.17 mJ/mm^2 (measurement of the manufacturer in accordance with the International Electrochemical Commission procedures) corresponding to a peak positive focal pressure of 27.5 MPa (275 bar) with a tensile value of the focal pressure of 6.6 MPa (66 Bar). The shock wave rise time and the pulse width were respectively 162 ns and 505 ns (data from the manufacturer). The total energy administered to cells for each treatment (E–6 dB) was of 3550 mJ

The spatial dimension of the ovoid shaped therapeutic focus measured 76.5 mm in length (fz– 6dB =76.5mm) and 17.3mm in diameter. To ensure the optimal treatment in relation to the focal dimensions, the flasks were placed at a distance

of 5 cm from the probe. Each cell population was treated with 1000 impulses at the frequency pulse of 3 Hz/sec.

At the end of the treatment, DMEM was removed and replaced with fresh complete medium; the culture flasks were then incubated in a normal horizontal position for 14 days. Untreated TCs were cultured in complete medium and used as controls. The experimental design is shown in Figure 1.

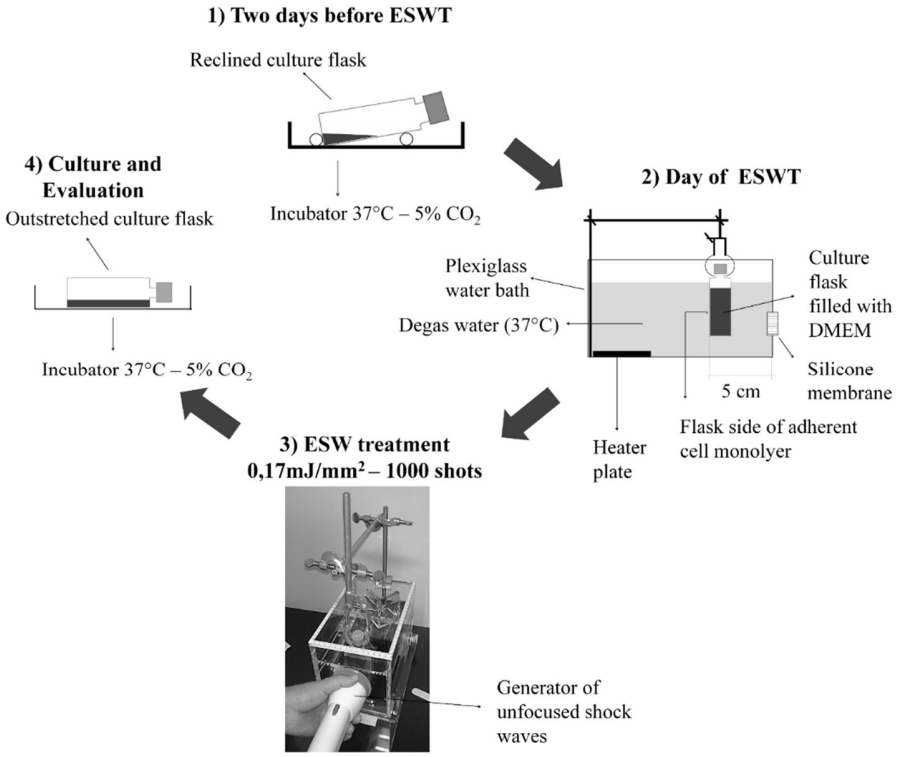

***Figure 1**.Experimental scheme representing the in-vitro shock wave treatment of adherent monolayer cell culture.*

Viability and Proliferation Assay

At 7 and 10 days after treatment, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, Sigma-Aldrich) was added to both the treated and the untreated cells at a final concentration of 0.5 mg/ml and incubated for 4 hours at 37°C. The resulting formazan precipitates were then solubilized using 100% DMSO and the absorbance was read at 570 nm (VictorX3, Perkin Elmer microplate, MA, USA) (Kingman et al. 2007). At the same time points, the treated and control cells were analyzed for DNA content (Triton X-100 0.1% in ddH_2O as lysis buffer) using the CyQUANT® Cell Proliferation Assay Kit (Invitrogen, Ltd., Paisley, UK); the fluorescence was read at 520 nm (excitation λ=480 nm) (VictorX3, Perkin Elmer microplate).

Cell apoptosis analysis by annexin V-FITC and propidium iodide

Apoptosis induced by SW treatment was analyzed by flow cytometry utilizing annexin V-FITC and propidium iodide (PI) staining (Sigma-Aldrich) at 2 and 7 days after ESWT. In brief, $4.0x10^5$ cells were trypsinized, washed with PBS and resuspended with 500 µL of a specific binding buffer containing 10 µL of PI, and 5 µL of annexin V-FITC (Kuypers et al. 1996; Pigault et al. 1994). After exactly 10 min of incubation in the dark at room temperature, the cells were analyzed for annexin V and PI staining by flow cytometry. Each experiment was run in triplicate. The excitation wavelength was 488 nm and emitted green fluorescence of annexin V (FL-1) and red fluorescence of PI (FL-2) which were collected using a 525 and a 575 nm band pass filter respectively. Early apoptosis and late apoptosis/necrosis were expressed as the percentages of annexin V+/PI− and annexin V+/PI+ positive cells.

RNA extraction, RT and real-time PCR

Total RNA was isolated from untreated and treated cells at 1, 2, 4 and 7 days after ESWT using the RNeasy Mini kit (Qiagen, Duesseldorf, Germany) and quantified at the spectrophotometer (Nanodrop, Thermo Scientific, Rockford, IL, USA). 100 ng of RNA were reverse-transcripted to cDNA employing the iScriptcDNA Synthesis Kit (Bio-Rad Laboratories, Benicia, CA, USA).

The final volume of 20 µL included a 5X reaction mix containing oligo(dT), random hexamer primers and reverse transcriptase pre-blended with RNase inhibitor. The reaction mix was incubated for 5 minutes at 25°C, 30 minutes at 42°C and 5 minutes at 85°C.

10 ng of cDNA were used as a template for real-time PCR, performed using a Rotor Gene RG3000 system (Qiagen). The PCR mixture included TaqMan Universal PCR Master Mix and Assays-on-Demand Gene expression probes (Life Technologies, Grand Island, NY, USA) in a final volume of 20 µL. Amplification and real-time data acquisition were performed using the following cycle conditions: 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. The analysed genes were: scleraxis (SCX) (Hs03054634_g1), type I collagen (COL1A1) (Hs01076777_m1), type III collagen (COL3A1) (Hs00943809_m1). The fold change in the expression of the different genes in the control and treated cells was normalized on the expression of the housekeeping gene glyceraldehydes-3-phosphate dehydrogenase (GAPDH) (Hs99999905_m1).

Cytokines, MMPs and VEGF determination in conditioned medium

The cumulative levels of soluble IL-1β, IL-6, IL-10, TNFα, TGFβ2, VEGF in the culture medium were determined at 0, 24 and 48 hours after the ESW treatment

by commercially available assays according to the manufacturers' instructions (R&D System, Minneapolis, USA). For the VEGF detection assay, the sensitivity of the test was 5 pg/mL, intra- and inter-assay coefficients of variation were 6.6% and 6.7%, respectively). For the IL-1β detection assay, the sensitivity of the test was less than 1 pg/mL, intra- and inter-assay coefficients of variation were 2.8% and 4.1%, respectively. For the IL-6 detection assay, the sensitivity of the test was 2 pg/mL, intra- and inter-assay coefficients of variation were 5.8% and 3.1%, respectively. For the IL-10 detection assay, the sensitivity of the test was less than 0.5 pg/mL, intra- and inter-assay coefficients of variation were 6.6% and 8.1%, respectively. For the TNFα detection assay, the sensitivity of the test was 1.6 pg/mL, intra- and inter-assay coefficients of variation were 5.0% and 7.3%, respectively. For the TGFβ2 detection assay, the sensitivity of the test was 2 pg/mL, intra- and inter-assay coefficients of variation were 2.7% and 4.3%, respectively.

Nitric Oxide (NO) quantification

At 2, 24 and 48 hours after ESW treatment, the culture medium of the treated and the untreated cells was collected to evaluate the cumulative NO synthesis. Nitrite ($NO2^-$), the stable end production of NO, was measured using modified Greiss reagent (Sigma-Aldrich). Greiss reagent was added in 1:1 ratio at the collected medium in a 96-well plate, then read at 550 nm with the spectrophotometer (VictorX3, Perkin Elmer microplate).

Statistical analysis

Statistical analysis was performed by GraphPad Prism v5.0 software (GraphPad Software Inc., LaJolla, CA, USA). All values are expressed as the mean ± SD. Normal distribution of values were assayed by Kolmogorov-Smirnov normality

test, while one-way Analysis of Variance (ANOVA) for repeated measures, with Bonferroni's correction, was used to compare data over time. Paired comparisons were performed by two-tailed t test. In the case of not normally distributed values, repeated measures were compared with the Kruskal-Wallis test with the Dunns' correction. Correlation analysis was performed by the two-tailed Pearson correlation test (Spearman's test for not normally distributed values); the same test was conducted to evaluate the correlation between the trends of these parameters across the time-points. The significance level was set at .05.

RESULTS

ESWT induced an increase in TCs viability and proliferation

The cell culture system that we adopted in this system allowed to reduce the cell growth surface in the flask, so that about 80% of tendon cells directly received the shock waves. Immediately after treatment, very small void areas were observed close to the center of focus region due to the detachment of few cells (Figure 2A).

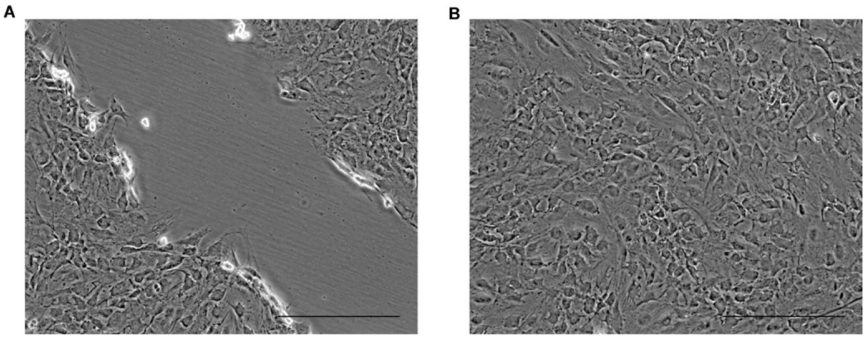

Figure 2*.Morphological appearance of TCs immediately after ESW treatment (A) and then the following day (B). ESWT initially causes cellular detachment, but 24*

hours later the monolayer is completely restored (optical microscopy 10X, scale bar 200 µm). Shape of void area is unrelated to the focus shape as only few cells were detached during the treatment.

Nevertheless, 24 hours later, the cell monolayer was completely restored (Figure 2B). Indeed, FACS analysis performed on ESW treated cells both at 2 and 7 days after treatment showed a similar apoptotic and necrotic rate to untreated cells, demonstrating that the ESWT was not cytotoxic (data not shown).

At day 7, cell viability significantly increased with respect to the untreated cells (+36%; p<.001) and this increase was maintained even after 14 days (+26%, p<.001) (n=7; Figure 3 A). At the same time points, treated TCs also showed a higher DNA content in comparison to untreated ones (+36% and +58% at 7 and 14 days, respectively), although these differences were not statistically significant (n=7; Figure 3 B).

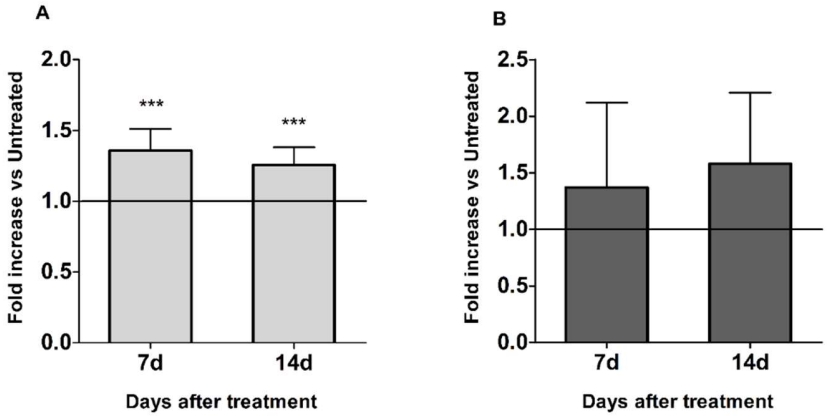

Figure 3*. Effect of ESW treatment on cell viability (A) and DNA content(B) of TCs after 7 and 14 days. Values are indicated asfold increase respect to untreated*

*cells represented by a fixed line set at 1; data are expressed as mean ± SD. ***p<.001, ESW-treated cells versus untreated cells, n=7.*

ESWT enhanced scleraxis and type I collagen gene expression

In order to evaluate the possible influence of ESWT on tendon specific gene expression, mRNA levels of scleraxis (SCX), type I collagen (COL1A1) and type III collagen (COL3A1) were analyzed at 1, 2, 4 and 7 days after treatment (n=5). The day after treatment, a statistically significant increase of SCX expression was observed in ESW treated cells in comparison to untreated ones (+52%; p<.05) (Figure 4A), although in the following time points SCX transcription progressively decreased. On the other hand, COL1A1 expression was up-regulated later: indeed, 4 days after ESWT, TCs showed higher levels of COL1A1 (+56%) with respect to untreated cells, but due to the high inter-donor variability this difference was not statistically significant (Figure 4B). Moreover, treated TCs showed a similar expression of COL3A1 to the untreated cells at all time points (Figure 4C), and also the ratio of COL3A1/COL1A1 expression did not change significantly in treated TCs (Figure 4D).

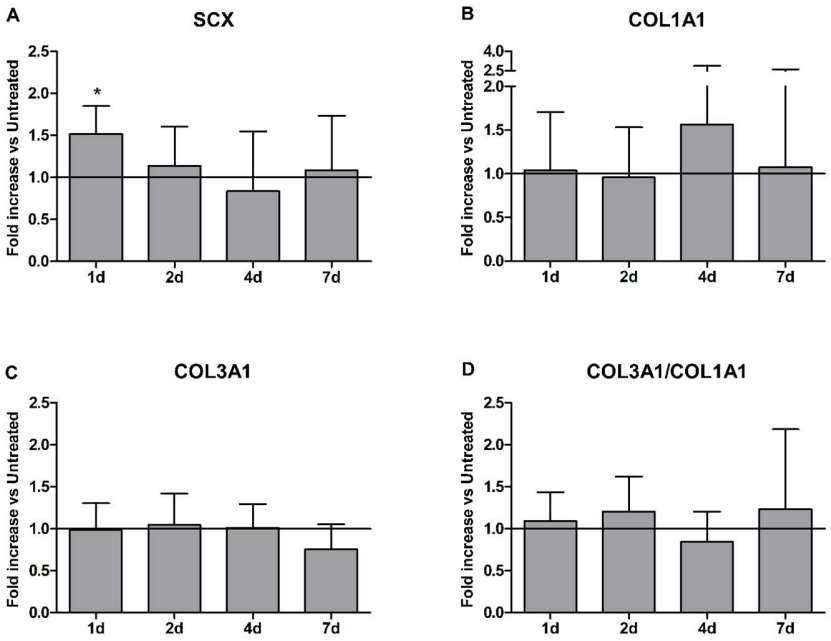

***Figure 4**. Effect of ESW exposure on scleraxis (SCX) (A), collagen type I (COL1A1) (B), collagen type III (COL3A1) (C) and COL3A1/COL1A1 ratio (D) gene expression, determined by quantitative real-time PCR at day 1, 2, 4 and 7 from the treatment. Values are the fold-increase of ΔΔCt respect to untreated cells represented by a fixed line set at 1, after normalization on housekeeping GAPDH gene expression. Significant modulation in gene expression was observed only for SCX, 1 day after treatment. Data are expressed as mean ± SD.*p<.05 ESW-treated cells versus untreated cells, n=7.*

Effect of ESWT on cytokines, growth factors and MMPs release

The level of pro- and anti-inflammatory cytokines, growth factors and matrix metalloproteinases released by TCs in the conditioned medium was evaluated at 0, 1, 2 and 7 days after ESWT (n=6). The release of IL-1β was significantly higher in treated cells at 1 day from ESWT with an increase of 571% ($p<.001$) (Figure 5A). Progressively, the IL-1β production decreased over time, even if statistically higher levels were still present at 7 days in the treated cells when compared to the untreated ones ($p<.001$). However, at all time points the absolute values of IL-1β are very low. On the contrary, TNFα levels were not significantly affected by ESW at any time points (Figure 5B). The release of IL-6 and IL-10 was also significantly higher in treated cells than in untreated ones. In particular, treated TCs released a significantly higher amount of IL-6 starting from day 1 after treatment (+504%, $p<.001$), and reaching a peak at day 2 (+ 641%, $p<.001$) (Figure 5C). Similarly, IL-10 production significantly increased after ESWT at any time point; in particular, the highest levels were observed in treated TCs compared to untreated ones at day 2 (+471%; $p<.001$) (Figure 5D). Moreover, a strong and significant release of TGFβ and VEGF in the culture medium of treated TCs was observed. Indeed, TGFβ release showed an 11-, 18- and 8-fold increase over the untreated cells at day 1, 2 and 7, respectively ($p<.001$; Figure 5E). VEGF production was even more evident and was 64-, 92- and 68-fold higher in the culture medium of the treated TCs at 1, 2 and 7 days after treatment, respectively ($p<.001$; Figure 5F).

There was no significant difference in the released level of MMP-3 and MMP-13 between the treated and the untreated cells at all-time points (Figure 6 A-B).

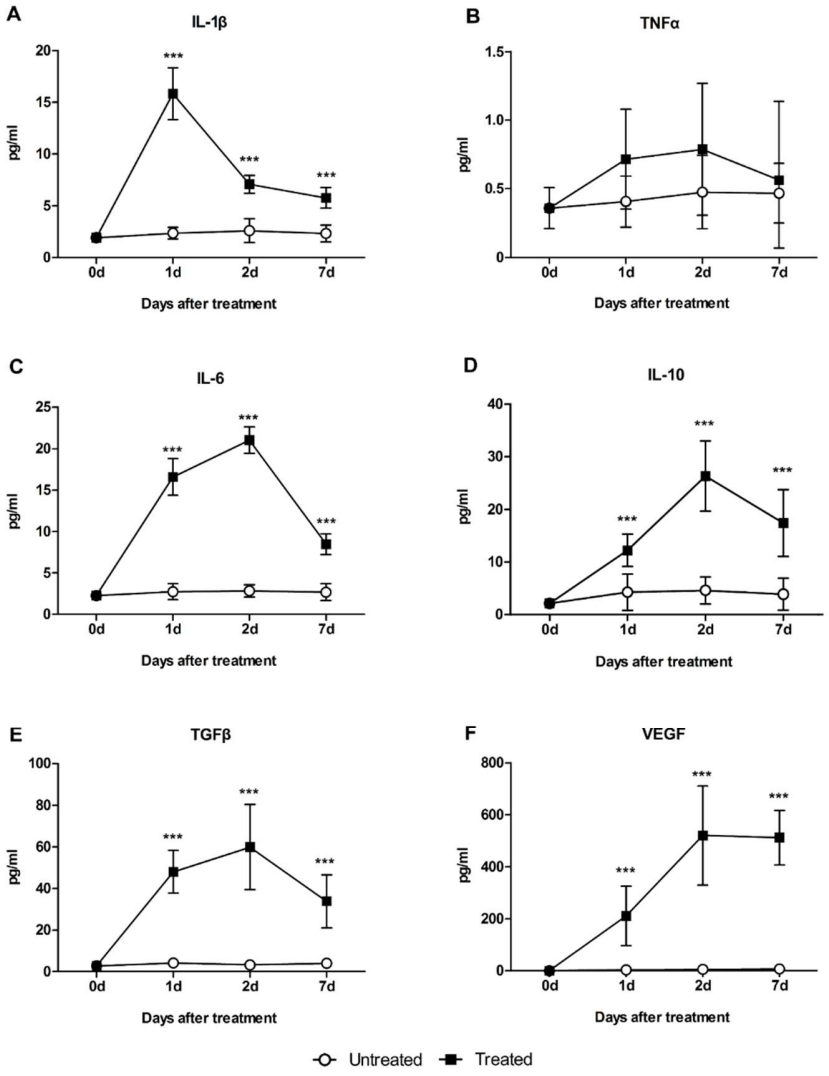

***Figure 5**. Release of cytokines and growth factors after 0, 1, 2 and 7 days from ESW exposure. TNFα (A) was not affected by ESWT, whilst the shock waves significantly increased the release of*

*IL-1β (B), IL-6 (C), IL-10 (D), TGFβ (E) and VEGF (F) in the culture medium in comparison to untreated cells.***p<.001 ESW-treated cells versus untreated cells, n=7.*

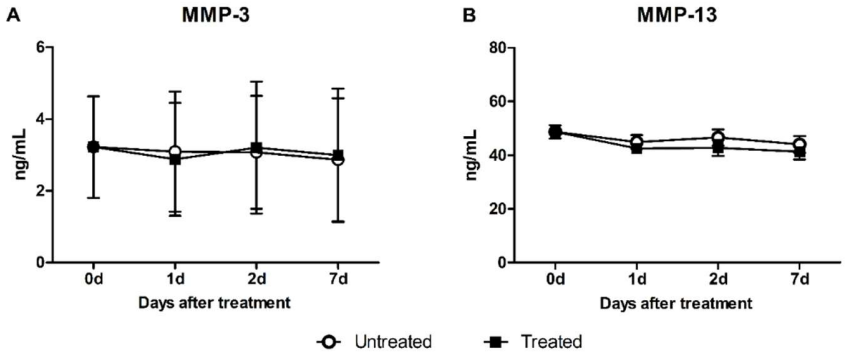

***Figure 6**. Release of MMP-3 (A) and MMP-13 (B) in the culture medium after ESW treatment. No differences were observed between treated and untreated cell at any time point, n=7.*

Nitrite evaluation

Cumulative nitric oxide (NO) production was assessed at 2, 24 and 48 hours after treatment in the TCs culture medium. Two hours after treatment the level of NO was undetectable in both the treated and the untreated medium (lower than 0.46 µM, which corresponded to the lowest point of the standard curve). Then, 48 hours after treatment the cumulative release of NO was significantly lower in the treated cells in comparison to the untreated ones ($p<.05$; Table I).

DISCUSSION

Our *in vitro* study showed for the first time the effects of soft-focused ESWT (0.17 mJ/mm^2) in a model of healthy human adherent tendon cells. The main findings of this paper highlights that ESWT positively influence cell viability and

proliferation of TCs and act modulating gene expression of tendon-specific markers, such as scleraxis, as well as the release of anti-inflammatory cytokines and of the growth factors TGFβ and VEGF.

A single *in vitro* report deals with the application of soft-focused shock waves on cells (Sansone et al. 2012) and no previous studies, to our knowledge, investigate the response of tendon cells to this kind of impulse. Pre-clinical studies have shown that soft-focused shock waves regulate the expression of chemokines (CXCL1, CXCL2, CXCL5), cytokines (IL-1β, IL-6, G-CSF, VEGF-A), matrix metalloproteinases (MMP-3, MMP-9, MMP-13), and the specific genes involved in the angiogenic pathway in a murine model of skin isografts, indicating a pro-angiogenic effect associated to the delay of inflammatory response (Stojadinovic et al. 2008). Further, in a murine model of full–thickness burn injury, soft-focused shock wave treatment reduced the inflammatory infiltrate and suppressed the expression of cytokines and MMPs (Davis et al. 2009), while in chronic wounds this treatment increased blood perfusion and tissue regeneration enhancing the expression of VEGF, eNOS and PCNA in fibroblasts (Kuo et al. 2009). Soft-focused shock waves have also been shown to be able to minimize the extension of the ischemic tissue necrosis and to induce angiogenesis, in ischemic heart failure (Mittermayr et al. 2011; Zimpfer et al. 2009). Finally, other studies have investigated the protective effect of soft-focused shock waves on the bone mass in experimental models of osteoporosis (Van der Jagt et al. 2009). With the exception of treatment of chronic wounds (Schaden et al. 2007), no other clinical applications of soft-focused ESWT have been reported to date.

For the first time, the effects of soft-focused ESW were analyzed on primary culture of human tendon cells while adhering to the culture plate. Our rationale

was to allow, already during ESW treatment, cell-cell contacts and interactions between cells and extracellular matrix, since these interplays represent a crucial point in mechanotransduction process. Indeed, physical forces influence conformational changes in membrane proteins, such as integrins, which result in intracellular signaling affecting gene expression and release of growth factors [Shyy et al. 1997, Skutek et al. 2001]. For these reasons our *in vitro* model allow to overcome the limitation of previous *in vitro* studies in which cells were treated in suspension (Chao et al. 2008, Vetrano et al. 2011, Leone et al. 2012). Since our findings in term of proliferation and scleraxis and type I collagen expression were comparable with those reported in these previous studies, the present investigation has to be considered as a further and strong confirmation of the ESW effect on tendon cells and, at the same time, it provides a more accurate model for the study of shock waves mechano-response in tendon cells. Furthermore, the similar acoustic impedance of the watery medium in the waterbath to that of human tissues, makes our model still closer to physiological conditions (Khun et al. 2008). A further advantage deriving from the use of soft-focused rather than focused shock waves on adherent cultures lies in the possibility of treating a larger area of cells. Indeed, in our model of adherent cell culture the traditional focused shock waves would have not reached an adequate number of cells, and thus the total biological effects would have been underestimated. Finally, since we did not use any specific technique to select only tenocytes, we treated a heterogeneous cell population, which better reflects the total cell population of a physiological tendon.

Our findings revealed that shock waves positively act on TCs proliferation and viability, both at 7 and 14 days after treatment, although the high interdonor

variability did not always allow to find statistically significant effects. These data are in agreement with some previous data showing that the effect of ESWT was observed no earlier than two weeks following shock wave exposure (Vulpiani et al. 2009; Speed 2004).

At the molecular level, the first interesting data regard the expression of scleraxis, a transcription factor specific for tenocytes and their progenitors. This protein, together with the transcription factor NFATc, regulates the expression of COL1A1 gene in tendon fibroblasts, and is part of the regulatory network involved in the differentiation of mesenchymal cells into fibroblasts (Lejard et al. 2007). Our data confirm this evidence: following the significant increase of scleraxis expression at one day after treatment, three days later we observed an up-regulation of the COL1A1 gene, although the increase was not significant. When compared to healthy tenocytes, higher levels of SCX and COL1A1 have been observed in tenocytes derived from pathological tendons (Leone et al. 2012), and in this study shock wave treatment provoked a decrease in their molecular levels. These apparently controversial observations confirm that SWs normalize the metabolic activities of cells.

Changes in the total collagen content and composition have been previously found in tendinopathy, including an increase in the proportion of types III and type V collagen, as well as an increase in the type III/type I collagen ratio (Riley et al. 1994; Lui et al. 2010; Maffulli et al. 2000; de Mos et al. 2007; Ireland et al. 2001). Our data showed no change both in collagen III and in the collagen III/collagen I ratio of expression after ESWT.

As in other tissues, tendon healing represents a multistep process delineated by different phases, mainly based on an inflammatory mechanism (Schulze-Tanzil

et al. 2004). Inflammation has been recently demonstrated to have an important role in the onset and maintaining of tendinopathies (Rees et al. 2013). So, because of the dual role of cytokines, both in tendon healing and in degenerative conditions, for the first time the ESW-mediated cytokines release by tendon cells was analyzed. These data assume an important implication as they could contribute to better explain the effects and the possible mechanism of ESW in the treatment of tendinopathy. The production of TNFα was not affected by the ESWT, whereas treated TCs released a significantly higher amount of IL-1β with respect to untreated cells, with a peak immediately after treatment (24h) and showing a decrease over time in culture. In addition to its important role in normal physiological events in tendon homeostasis and repair, IL-1β is also a stimulus for the production of metalloproteases (MMPs), considered to be responsible for the extracellular matrix (ECM) degradation and, consequently, of the degeneration of the tendon (Archambault et al. 2002; Clegg et al. 2007). For this reason we also evaluated whether the increase of IL-1β corresponded to the production of MMP-3 and MMP-13, implied in the de-structuring of the bundles of the collagen fibers (Archambault et al. 2002; Tsuzaki et al. 2003). Both MMP-3 and MMP-13 were barely detectable after ESWT, suggesting that the increased level of IL-1β released by TCs after ESWT was not correlated to the degradation of the ECM. So, in this case, the increase of IL-1β should be interpreted as the primer for the consequent increase of IL-6, which in turn promoted the increase of IL-10, showing a peak after 48 hours. This pathway perfectly agrees with the healing inflammatory mechanism, where the initial acute response is followed, about 48 hours after the stimulus, by the production of IL-10, an anti-inflammatory cytokine responsible for the self-resolving phase of inflammation

(Schulze-Tanzil et al. 2004). Similarly, TGFβ, a growth factor with anti-inflammatory properties, showed a strong increase 48h after ESWT. Taken together these results indicate that ESWT was able to induce the initial, beneficial inflammatory phase of the tissue healing mechanism. Experimental models of mechanotransduction have shown a significant correlation between physical forces and TGFβ and SCX expression (Maeda et al. 2011). In particular, it has been shown that mechanical forces regulate the expression of SCX through activation of a TGFβ/Smad mediated pathway which acts in the maintenance of ECM integrity.

TGFβ is known to be involved in other phases of tissue healing, such as cell proliferation, cell viability and stimulation of collagen production (Zhu and Burgess 2001). Growth factors are among the most important molecules involved in the healing process, but their specific actions are not well characterized. Wang et al. reported that the transfer of exogenous VEGF gene to proliferating tenocytes increased the expression of the endogenous TGFβ (Wang et al. 2005). It is likely that these factors work synergistically, playing an integral role in the tendon healing process. However, the role of VEGF in tendon healing is not completely understood. Indeed, although VEGF induction is necessary for tissue repair, it has been also associated with sustained elevation of tendon pathology (Molloy et al. 2003; Pufe et al. 2005). It has been observed that increased levels of VEGF within an injury site are correlated with a well-defined pattern of vascular ingrowth from the epi- and intra-tendinous blood supply toward the site of repair providing extrinsic cells, nutrients, and growth factors to the injured area (Molloy et al. 2003). On the other hand, there is also evidence that VEGF-induced angiogenesis might be correlated with degenerative tendon

disease inducing MMPs expression and inhibition of TIMPs (Pufe et al. 2005). One of the main reported effects of ESWT treatment in various tissues (although not yet in tendon tissue) is the neo-angiogenesis promoted by VEGF (Wang et al. 2003; Wang 2003a; Mittermayr et al. 2011; Furia et al. 2010; Ito et al. 2009). In our study, ESWT induced a marked increase of VEGF starting from 48h after the treatment. This increase can be explained by the fact that IL-6 and IL-10 are the main cytokines stimulating VEGF production (Tartour et al. 2011). Together with the production of inflammatory cytokines and growth factors, such as TGFβ, the significant production of VEGF indicates that ESWT has a profound influence on several aspects of the tendon healing process, including neo-angiogenesis.

Besides inflammatory cytokines, growth factors, and other factors, part of the cell-to-cell and the cell-to-matrix interactions is mediated by some soluble mediators, including NO (Schäffer et al. 2002, Hwang et al. 2000). Contrary to other authors (Chao et al. 2008) who have demonstrated that ESWT promotes the release of NO, we observed significantly lower levels of NO in ESW-treated cells with respect to untreated ones. These differences can probably be explained by the different protocol we used; it has been shown that the response of cells to shock waves can depend on the type of generator used, as well as on different energy settings and the type of shock waves produced (Martini et al. 2006). Indeed, in their study Chao et al. (2008) treated rat tenocytes with very high EFD shock waves (0.36 mJ/mm^2) using the traditional cell suspension model. They observed an increase of NO only when cells were treated with a low number of impulses (≤100). The differences in experimental protocol make it difficult to compare our study to that of Chao. However, since we observed significant effects of ESWT on TCs, it is possible that these shock wave–related adaptive

changes may, at least in part, be controlled by a process in which mediator(s) other than NO, may play a pivotal role. For example, the physical forces could affect cytoskeleton through mechanotransduction inducing activation of membrane proteins (integrins and receptors coupled to G-proteins) that could be responsible of cell responses observed in our study, such as the increase of TGFβ production. Indeed, it is known that this growth factor is able to stimulate migration, proliferation and collagen synthesis in tendon cells (Tsai et al. 2011). It would be important to verify the hypothesis that soft-focused shock waves could activate tendon progenitor cells from their niches, starting from our experimental model. This could prefigure a new rationale for routinely employing soft-focused shock waves to treat the failed healing status which distinguishes tendinopathies.

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Aim 2

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4.3 Manuscript 6: *In vitro* functional response of human tendon cells to different dosages of low frequency pulsed electromagnetic field

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ABSTRACT

Purpose: Chronic tendinopathy is a degenerative process causing pain and disability. Current treatments include biophysical therapies, such as pulsed electromagnetic fields (PEMF). The aim of this study was to compare, for the first time, the functional *in vitro* response of human tendon cells to different dosages of PEMF, varying in field intensity, duration and number of exposures.

Methods: Tendon cells, isolated from human semitendinosus and gracilis tendons (hTCs; n=6), were exposed to different PEMF treatments (1.5mT or 3mT for 8 or 12 hours, single or repeated treatments). SCX, COL1A1, COL3A1 and VEGF-A expression and cytokine production were assessed.

Results: None of the different dosages provoked apoptotic events. Proliferation of hTCs was enhanced by all treatments, whereas only 3mT-PEMF treatment increased cell viability. However, the single 1.5mT-PEMF treatment elicited the highest up-regulation of SCX, VEGF-A and COL1A1 expression, and it significantly reduced COL3A1 expression with respect to untreated cells. The treated hTCs showed a significantly higher release of IL-1β, IL-6, IL-10 and TGFβ. Interestingly, the repeated 1.5mT-PEMF significantly further increased IL-10 production.

Conclusions: 1.5mT-PEMF treatment was able to give the best results in *in vitro* healthy human tendon cell culture. Although the clinical relevance is not direct, this investigation should be considered an attempt to clarify the effect of different PEMF protocols on tendon cells, in particular focusing on the potential applicability of this cell source for regenerative medicine purpose, both in surgical and in conservative treatment for tendon disorders.

Abbreviations: Pulsed electromagnetic field (PEMF), human Tendon Cells (hTCs)

Keywords: Pulsed electromagnetic field, PEMF dosage, human Tendon Cells, Tendon markers, Anti-inflammatory action

INTRODUCTION

Tendon disorders are a class of pathology, which includes traumatic injuries as well as chronic diseases, such as tendinopathy. The recovery from these tendon pathologies can be long and is often not successful. In particular, the mechanisms underlying tendinopathy, whether chronic or acute, are still partially unclear. In fact, the debate about the possible role of inflammation in this pathology still divides clinicians and researchers. Recently, new findings have demonstrated the presence of inflammatory elements in chronic tendon disease, such as the infiltration of lymphocytes and macrophages in the peritenon, the activation of matrix metalloproteinases, and the involvement of mediators like substance P, vascular endothelial growth factor (VEGF) and cycloxigenase type II (COX2) [26]. Pulsed electromagnetic fields (PEMF) could be an innovative approach in the management of tendon disorders [9]. In the last few years PEMF have been demonstrated to be effective also in the treatment of several different pathologies, such as bone delayed union or non-union and articular cartilage disease, including early osteoarthritis [1,12,15,20,34]. *In vitro* studies have shown that PEMF can limit the catabolic effects of pro-inflammatory cytokines on articular cartilage and promote anabolic activity of the chondrocytes [6,25] thus suggesting that they may be used to control articular tissue inflammation and to stimulate anabolic pathways, eventually resulting in tissue repair [2,36]. It has also been showed that PEMF are able to increase A_{2A} and A_3 adenosine receptor density and functionality in different cell lines, human neutrophils,

chondrocytes and synoviocytes, resulting in the decrease of pro-inflammatory cytokines, such as IL-6 and IL-8, and on the inhibition of prostaglandin E2 (PGE_2) [23,38,40], and of NFkB and FGF-2 release [24,30]. Moreover, the existence of electromagnetic-responsive DNA sequences, at least in the Hsp70 promoter, suggests that PEMF could directly modulate expression of specific proteins [29]. At cellular level PEMF have been proved to be effective in the modulation of cell proliferation, gene expression and cytokine production also in human tendon cells (hTCs) [4,10,35]. However, in order to propose the optimum PEMF treatment protocol for each pathology, the conditions that obtain the most efficient dose-response effect must be defined. *In vitro,* physical-dynamic studies would allow researchers to find not only the best cell response, but also the minimal effective dose both in terms of daily exposure and of field intensity that could be predictive of the *in vivo* situation [4].

It is noteworthy that no previous *in vitro* studies on tendon cells have taken into account the possible different biological effects exerted by different PEMF intensity and different length of exposure. For this reason, the aim of this study was to evaluate for the first time the relationship between PEMF dosage and *in vitro* response of hTCs, comparing the effects of a low intensity PEMF exposure (1.5mT- PEMF) against double intensity (3mT-PEMF), and with a repetition of the same treatment (R-1.5mT-PEMF), using two different lengths of exposure (8 and 12 hours). The results of this study could contribute to comprehend the effect of PEMF on tendon cells and thus on tendon disorders.

MATERIALS AND METHODS

hTC isolation

Tendon cells were isolated from the discarded portion of healthy semitendinosus and gracilis tendons of 6 donors (mean age 35±12 years) after anterior cruciate ligament reconstruction by hamstring technique at Galeazzi Orthopaedic Institute, under written consent. The tendon portions were minced and enzymatically digested overnight at 37°C with 0.3% collagenase type I (Worthington, Lakewood, NJ, USA) in DMEM (Sigma-Aldrich, St. Louis, MO, USA) (modified from [31]). The resulting nucleated cells were plated $5x10^3$cells/cm^2, in complete medium composed of DMEM, 10% fetal bovine serum (FBS, Sigma Aldrich), 50 U/mL Penicillin, 50 U/mL Streptomycin, 2 mM L-glutamine (Sigma Aldrich) and supplemented with 5 ng/mL basic human fibroblast growth factor (bFGF; Peprotech, Rocky Hill, NJ, USA) for the cell expansion phase only and maintained in culture. When cells reached 80-90% confluence they were detached by trypsin-EDTA (0.5% trypsin/0.2% EDTA; Sigma-Aldrich) and cultured at a density of $3x10^3$ cells/cm^2. The cells were used for experiments at passage 4.

PEMF stimulation

PEMF were generated using a pair of rectangular horizontal coils as previously described [19]. The peak intensity of the magnetic field between coils was 1.5 or 3 ± 0.2mT, measured using the Hall probe (HTD61-0608-05-T, F.W. Bell, Sypris Solutions, Louisville, KY, USA) of a gaussmeter (DG500, Laboratorio Elettrofisico, Milan, Italy) with a reading sensitivity of 0.2%. Cells were plated the day before stimulation and exposed to a single PEMF treatment at 1.5mT and 3mT field intensity (1.5mT-PEMF, 3mT-PEMF) or for three 1.5mT-PEMF (R-1.5mT-PEMF) treatments with an interval of 48 hours between the start of each exposure. For all the conditions both 8 and 12 hours of exposure were tested (Table 1).

Live and Dead Assay

Untreated and treated cells were analyzed by live/dead assay. Cells were seeded at a density of 10^5 cells/cm^2. Immediately after the end of treatment, the culture medium was removed and the cells were incubated with a solution of 2 µM calcein and 4 µM ethidium homodimer-1 (Invitrogen Ltd., Paisley, UK). The samples were observed by fluorescence microscope (OLYMPUS IX71). The percentage of live and dead cells was defined as PLive = NLive/(NLive+NDead), where NLive is the number of live cells and NDead of dead cells in the same image. For each population three different randomly selected fields of 2 replicate samples were analyzed.

Viability and Proliferation Assays

Passage 4 hTCs were plated at a density of $1.5x10^4$ cells/cm^2 in 96-well plates and exposed to PEMF as previously described. Measures of cell viability were performed at 0 and 2 days after the end of treatments, adding a final concentration of 0.5 mg/mL MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, Sigma-Aldrich) to the culture medium and incubating for 4 hours at 37°C. After medium removal, 100% DMSO was then added to each well. Absorbance of this solution was read at 570 nm (VictorX3, Perkin Elmer microplate, Waltham, MA, USA) [17]. DNA content analysis was performed on cells plated in 24-well plates at a density of $5x10^3$ per cm^2, at 0 and 2 days after the end of treatment using CyQUANT® Cell Proliferation Assay Kit (Invitrogen Ltd.);; fluorescence was read at 520 nm (excitation λ= 480 nm) (VictorX3, Perkin Elmer microplate).

Gene expression analysis

RNA from each sample was obtained using A RNeasy Mini kit (Qiagen, Dusseldorf, Germany) and quantified spectrophotometrically (Nanodrop, Thermo Scientifc, Rockford, IL, USA). 120 ng of RNA was reverse transcripted to cDNA with iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Benicia, CA, USA) in a final volume of 20 µL, with 5x reaction mix containing random hexamer primers, oligo(dT), and reverse trascriptase pre-blended with RNase inhibitor. The reaction mix was incubated at 25°C for 5 minutes, at 42°C for 30 minutes, and at 85°C for 5 minutes. Real time PCR mixture was made by 10 ng cDNA template, TaqMan Universal PCR Master Mix, and Assays-on-Demand Gene expression probes (Life Technologies, Grand Island, NY, USA). The reaction was performed with Applied Biosystems Step One Plus (Life Technologies) in a final volume of 20 µL. The following cycle conditions were used to perform amplification and real-time data acquisition: 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles at 95°C for 15 seconds, and 60°C for 1 minute. Glyceraldehydes-3-phosphate dehydrogenase (GAPDH) (Hs99999905_m1), scleraxis (SCX) (Hs03054634_g1), collagen type I (COL1A1) (Hs01076777_m1), collagen type III (COL3A1)(Hs00943809_m1) and vascular endothelial growth factor-A (VEGF-A) (Hs00900055_m1) genes were analyzed. The fold change in expression of analyzed genes in treated and untreated samples was normalized on expression of the GAPDH housekeeping gene. Data are expressed as fold increase on the respective controls for each gene and sample.

Cytokine and growth factor determination

The cumulative amount of soluble IL-1β, IL-6, IL-10, TNF-α, TGFβ2 released by cells in the culture medium was determined at 0, 24 and 48 hours after the end

of PEMF treatment by commercially available ELISA assays, according to the manufacturers' instructions (R&D System, Minneapolis, USA). For the IL-1β detection assay, the sensitivity of the test was less than 1 pg/mL, intra- and inter-assay coefficients of variation were 2.8% and 4.1%, respectively. For the IL-6 detection assay, the sensitivity of the test was 2 pg/mL, intra- and inter-assay coefficients of variation were 5.8% and 3.1%, respectively. For the IL-10 detection assay, the sensitivity of the test was less than 0.5 pg/mL, intra- and inter-assay coefficients of variation were 6.6% and 8.1%, respectively. For the TNF-α detection assay, the sensitivity of the test was 1.6 pg/mL, intra- and inter-assay coefficients of variation were 5.0% and 7.3%, respectively. For the TGFβ2 detection assay, the sensitivity of the test was 2 pg/mL, intra- and inter-assay coefficients of variation were 2.7% and 4.3%, respectively.

The study was approved by the Institutional Review Board of the Galeazzi Orthopaedic Institute (Ref. Number IOG-2.47).

Statistical Analysis

Statistical analysis was performed by GraphPad Prism v5.0 software (GraphPad Software Inc., La Jolla, CA, USA). All values are expressed as the mean ± SD. Normal distribution of values were assayed by Kolmogorov–Smirnov normality test, while one-way Analysis of Variance (ANOVA) for repeated measures, with Bonferroni's correction, was used to compare data over time. Paired or unpaired comparisons were performed by two-tailed t test. In the case of not normally distributed values, repeated measures were compared with the Mann-Whitney test. p values <0.05 were considered statistically significant.

RESULTS

hTCs treated with 1.5mT- and 3mT-PEMF showed a similar cell viability and DNA content

As shown by Live&Dead assay, no combination of treatment provoked any cell death event, as in all cases more than 98% of cells were viable, without any differences between untreated and treated hTCs (Fig. 1).

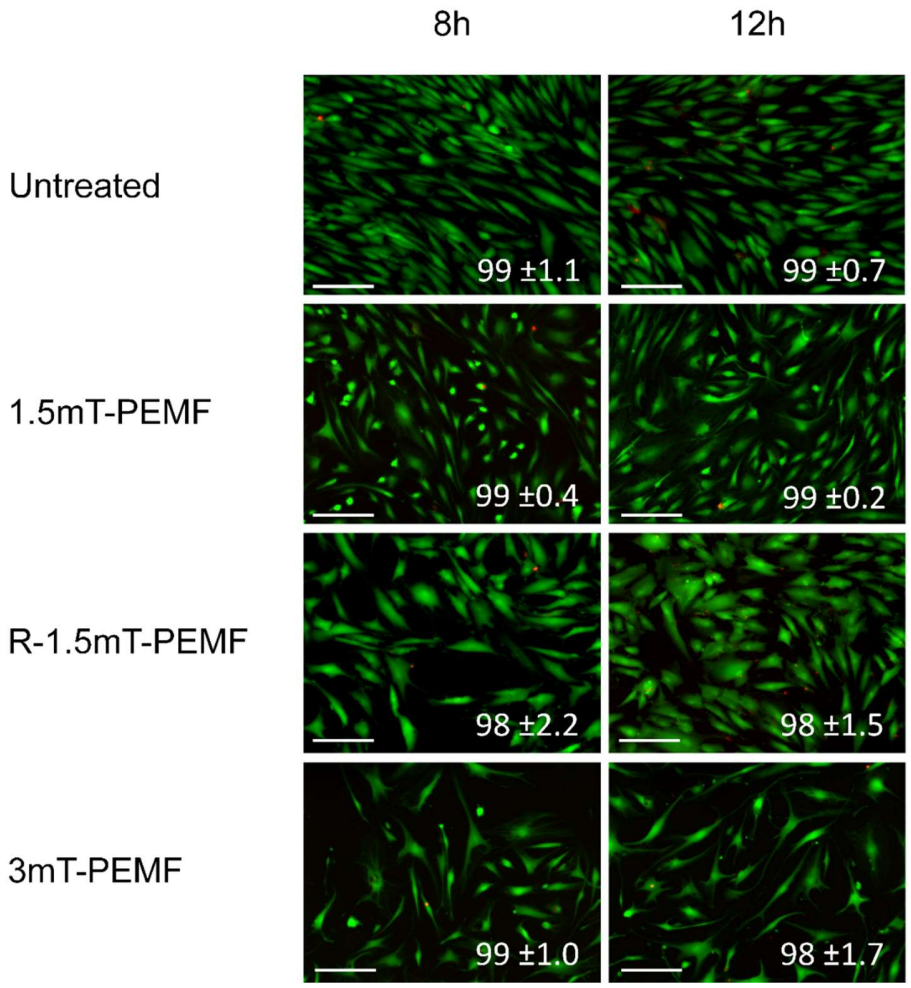
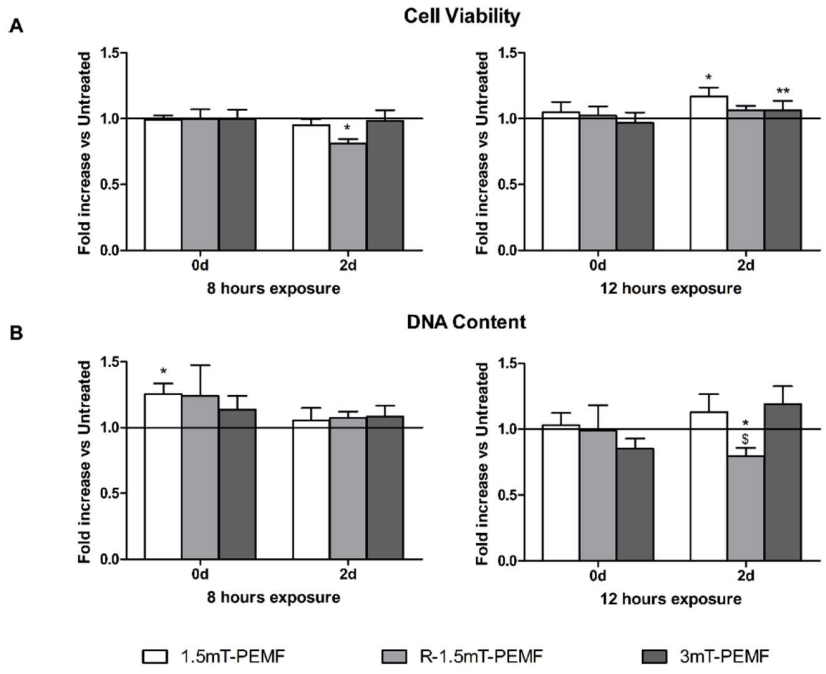


***Fig 1**. Live&Dead staining of untreated and PEMF treated cells for 8h and 12h (green, viable cells; red, dead cells). Percentages of viable cells (expressed as mean ± SD) are reported for each group (fluorescence microscopy, scale bar 200 µm, merged images).*

The viability of hTCs exposed to PEMF treatments was also confirmed by MTT assay; The only decrease was observed in 8h-R-1.5mT-PEMF treated cells at 2 days with respect to untreated cells (-19%, $p<0.05$) (Fig. 2A). On the other hand, all the samples treated for a longer time period (12 hours) showed an increase of hTC viability with respect to untreated cells at day 2, with statistically significant increases for the 1.5mT- and 3mT-PEMF treatments (+17%, $p<0.05$ and +13%, $p<0.01$, respectively) (Fig 2A). Indeed, immediately after 8h treatment (0 day), 1.5mT-PEMF treated cells, as well as R-1.5mT-PEMF and 3mT-PEMF hTCs showed an increase in DNA content of 25%, 24% and 14% with respect to untreated cells, although only the first one was significant ($p<0.05$; Fig 2B). At 2 days, all treated cells showed a DNA content comparable to that of the untreated ones. A divergent behavior was observed when cells were exposed to the different PEMF treatments for 12 hours: at day 0 the DNA content of hTCs was not affected by the stimulation, whereas after 2 days both 1.5mT- and 3mT-PEMF induced an increase with respect to the untreated cells, although it was not significant. Conversely, the repeated stimulation (R-1.5mT-PEMF) induced, at 2 days, a significant decrease of DNA content with respect to untreated cells (-25%, $p<0.05$).



hTCs treated with a single 1.5mT-PEMF treatment showed higher VEGF-A, SCX, COL1A1, and COL1A1/COL3A1 ratio transcript levels

SCX expression seemed to be positively influenced in a dose-dependent manner by the length of exposure for all types of treatments, in particular for the 1.5mT-PEMF treatment (8 hours, +54%, $p<0.05$; 12 hours +79%, n.s.) (Fig. 3A). Non-significant slight increases were observed for R-1.5mT- and 3mT-PEMF treatments after 12 hours of exposure at day 0 (+12% and +10%, respectively). Comparing the three groups of treatment, hTCs treated for 8 hours with R-1.5mT-PEMF and assessed at day 0 showed a significantly lower SCX expression in comparison to that observed in 1.5mT-PEMF treated cells (-46%, $p<0.05$).

1.5mT-PEMF treatment was able to significantly up-regulate VEGF-A expression at day 0 (8h, +100%, $p<0.05$; 12h: +75%, $p<0.05$), while other PEMF treatments did not significantly affect it. Cell treated for 8 and 12 hours showed a similar trend, with just slightly higher increases of VEGF-A expression at day 0 after 12 hours compared to 8 hours (Fig. 3B).

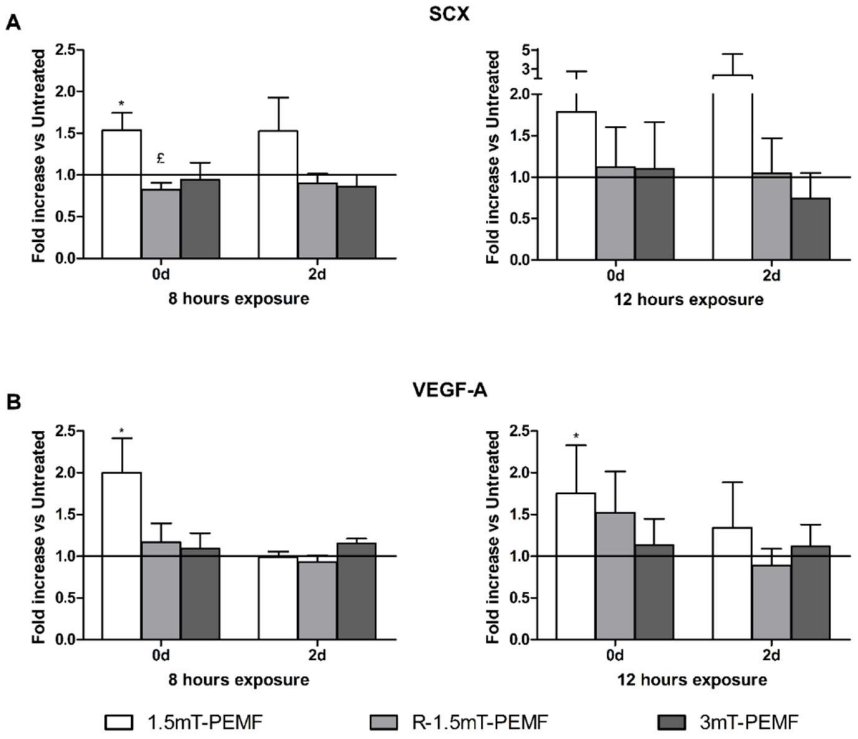


***Fig 3**. Gene expression of SCX (A) and VEGF-A (B) at 0 and 2 days after 8h and 12h from PEMF treatments (n=6), assessed by Real time PCR. Data are expressed as average fold increase ± SD respect to untreated samples (fixed line set at 1),*

normalized on GAPDH expression. Levels of significance: $^{*}p<0.05$ vs untreated cells; $^{£}p<0.05$ vs 1.5mT-PEMF.

Amongst the different PEMF treatments, only 1.5mT-PEMF was able to up-regulate COL1A1 expression with respect to untreated cells at least at day 0, both after 8h (+140%) and 12h (+133%), although, due to the high interdonor variability, this increase was not statistically significant (Fig. 4A). hTCs exposed to R-1.5mT-PEMF for 8 hours showed a significantly lower COL1A1 expression in comparison to 1.5mT- and 3mT-PEMF treated ones (-34% and -42%, respectively, both $p<0.05$). The hTCs exposed to 3mT-PEMF treatment behaved differently, since a slight up-regulation of COL1A1 expression with respect to untreated cells (+46% and +22% for 8 and 12 hours, respectively, n.s) was observed only 2 days after treatment.

Interestingly, PEMF treatments did not significantly affect the hTC COL3A1 expression, with the exception of those exposed for 8 hours to a single 1.5mT-PEMF treatment: indeed, at day 0, a significant reduction of collagen type III expression with respect to untreated cells was observed (-50%, $p<0.05$; FIG4B). In general, the resulting COL1A1 /COL3A1 expression ratio was higher in all the PEMF treated cells compared to untreated ones, with the exception of those exposed to R-1.5mT-PEMF. In particular, it was much higher in the single 1.5mT-PEMF treated cells for both 8h (+218%, n.s. and +47%, $p<0.05$ at 0 and 2 days, respectively) and 12h (+181% and +143% at 0 and 2 days, respectively, both n.s.) with respect to untreated cells (data not shown).

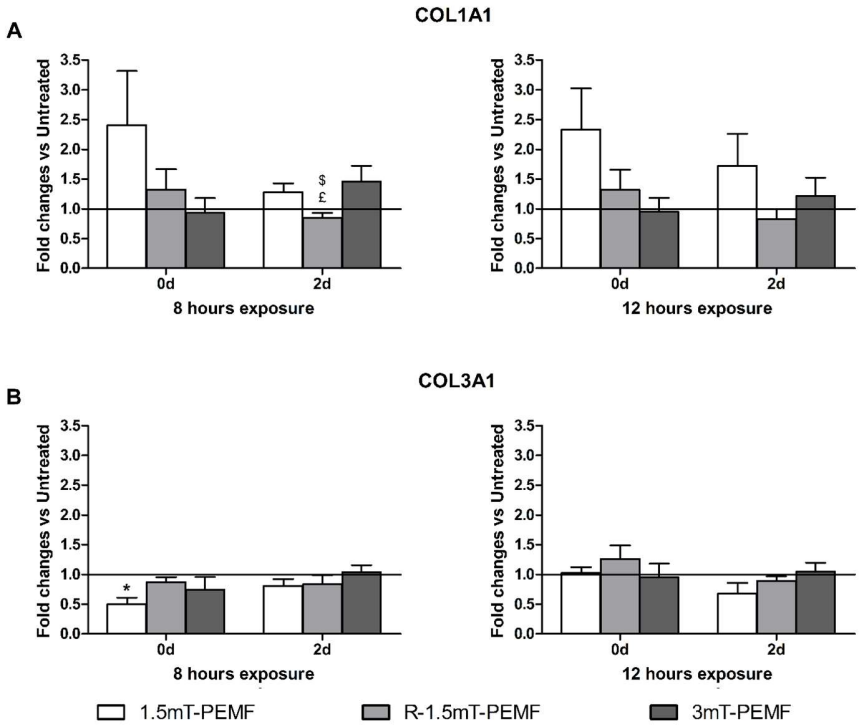


***Fig 4**. Gene expression of COL1A1 (A) and COL3A1 (B) at 0 and 2 days after 8h and 12h PEMF treatments (n=6), assessed by Real time PCR. Data are expresses as average fold increase ± SD respect to untreated samples (fixed line set at 1), normalized on GAPDH expression. Levels of significance: $^{*}p<0.05$ vs untreated cells; $^{£}p<0.05$ vs 1.5mT-PEMF; $^{\$}p<0.05$ vs 3mT-PEMF*

R-1.5mT-PEMF treatment induced a higher IL-10 release

All the PEMF treatments were able to increase the production of IL-1β in hTCs at each time point in a field intensity-dependent manner (FIG. 5A). However, at day 0 the increase of IL-1β, both after 8 and 12 hours of exposure to 1.5mT-PEMF

single treatment, was not significant in comparison to untreated cells. In general, when the cells were treated for 8 hours, the maximum levels of IL-1β were observed one day after the exposure, and then reduced over time. Conversely, when cells were treated for a longer time, 12 hours, the IL-1β release was significantly higher in comparison to the amount released by untreated cells, starting immediately at the end of the treatment up to 2 days.

TNFα release was only slightly modulated by all the PEMF treatments, with the exception of 3mT-PEMF and of R-1.5mT-PEMF treatments, which were able to significantly up-regulate its release after 8 hours of exposure at 2 days (+17%, $p<0.05$) and after 12 hours at 1 day (+30%, $p<0.05$), respectively (FIG.5B).

The release of IL-6 by hTCs was significantly up-regulated by all the PEMF treatments and at each time point (FIG.5C). Very similar levels, as well as temporal trends, were observed after 8 and 12 hours of exposure; indeed, in both cases, IL-6 was immediately released by the cells (0 day), then reached the maximum and stable levels one day after treatment. R-1.5mT PEMF treated cells seemed to be less affected by the treatment in terms of IL-6 production, in particular at 2 days after 12 hours of exposure, although their amount of IL-6 production was always significantly higher than the controls.

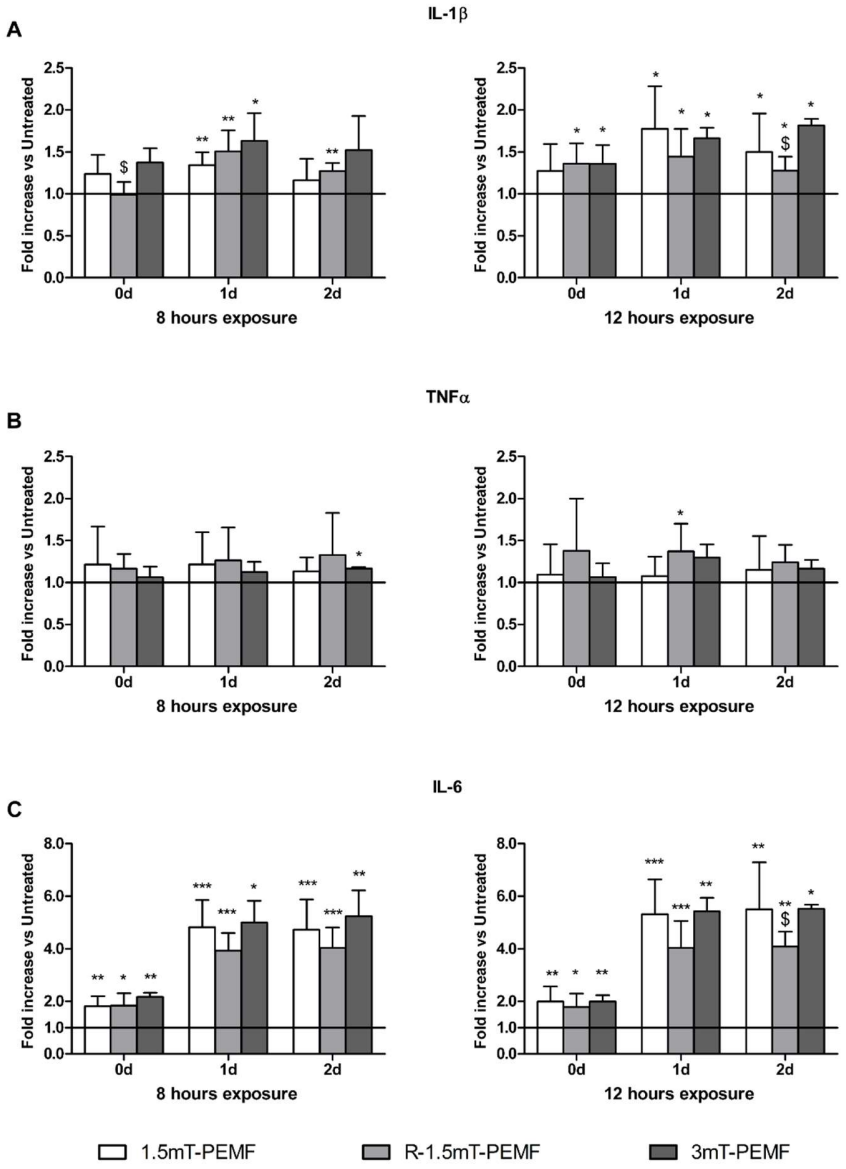
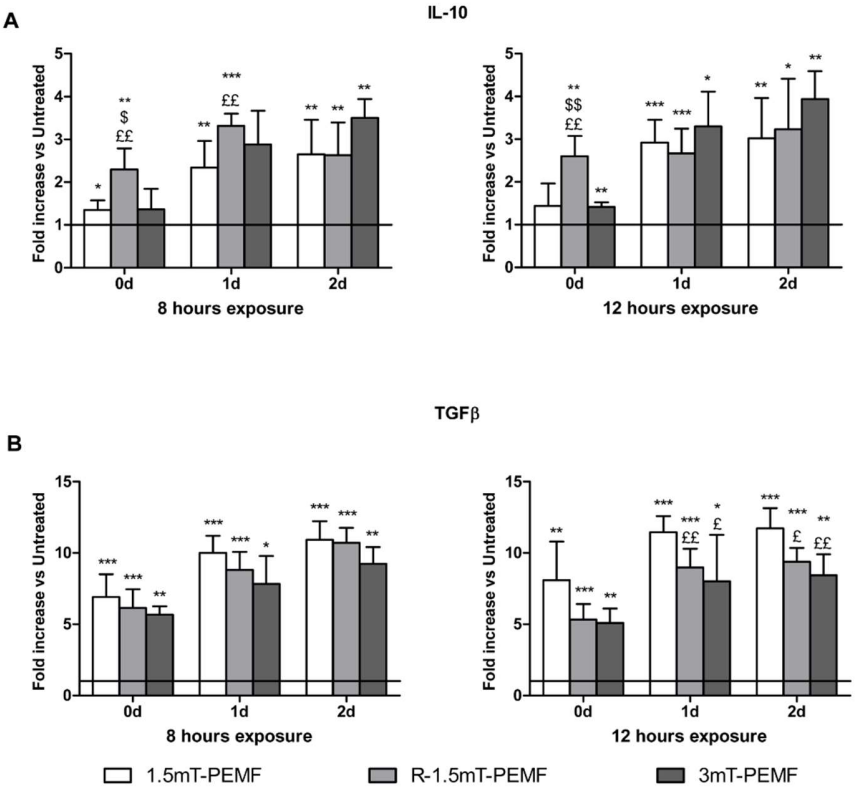


Fig 5. *IL-1ß (A), TNFα (B), and IL-6 (C) production in hTCs at 0, 1, and 2 days from PEMF treatments (n=6), assessed by ELISA assay. Data are expressed as average*

fold increase ± SD respect to untreated cells (fixed line set at 1). Levels of significance: $^{*}p<0.05$,$^{**}p<0.01$,$^{***}p<0.001$ vs untreated cells; $^{\$}p<0.05$ vs 3mT-PEMF.

A similar scenario was observed for IL-10: also in this case all the PEMF treatments were able to increase the production of this cytokine by hTCs, with increases ranging from +35% to +300% in comparison to untreated cells, and no significant differences were found between cells treated for 8 and 12 hours (FIG. 6A). However, diverging from our observations for IL-6, hTCs treated with R-1.5mT PEMF showed the highest increase in terms of IL-10 release in comparison to the other treated cells, particularly at day 0, both after 8 and 12 hours of exposure (+70% and 80% vs 1.5mT-PEMF for 8 and 12 hours respectively, $p<0.01$ and +68% and +83% vs 3mT-PEMF for 8 and 12 hours respectively, $p<0.05$).

Also TGF-β levels were found to be significantly higher in all groups at all-time points, with fold increases ranging from 6 to 11 with respect to untreated ones (FIG.6B) and with the highest release of this growth factor was observed in cells exposed to a single 1.5mT-PEMF treatment; this effect was particularly evident after 12 hours of exposure, when, at both 1 and 2 days after treatment, 1.5mT-PEMF treated cells showed a significantly higher amount of TGF-β with respect to both 12 hours R-1.5mT- (1 day, +27%, $p<0.001$; 2 days, +25%, $p<0.05$) and 3mT-PEMF (1 day, +43%, $p<0.01$ and 2 day ,+39%, $p<0.05$, respectively).



6. *IL-10 (A) and TGFβ (B) production in hTCs at 0, 1, and 2 days after different PEMF treatments (n=6), assessed by ELISA assay. Data are expressed as average fold increase ± SD respect to untreated cells (fixed line set at 1). Levels of significance: *p<0.05,**p<0.01,***p<0.001 vs untreated cells; £p<0.05, ££p<0.01 vs 1.5mT-PEMF; $p<0.05, $$p<0.01 vs 3mT-PEMF.*

DISCUSSION

The most important finding of the present study is that the length of exposure, the field intensity and the number of PEMF treatments differently affect tendon

cell proliferation, gene expression and release of pro- and anti-inflammatory cytokines. Our results could improve knowledge about the role of PEMF in the treatment of tendon pathologies. Indeed, to date only a few studies on the clinical effect of PEMF on tendon or ligament repair have been reported [9] and these are sometimes conflicting, probably due to the different PEMF dosages used. Moreover, from the basic science point of view, the mechanism and effects of PEMF are far from being clearly defined, even if the cell membrane is often considered to be their main target [21], as they could affect membrane mediated signal transduction processes, modulating cell proliferation, gene expression, and activity of cytokines and growth factors involved in the inflammatory response and in tissue repair, such as FGF-2, VEGF, NFkB, and IL-1β [3,30,39,40,41,43]. Recently it has been observed that a single exposure to PEMF positively influences gene expression of tendon-specific markers and the release of anti-inflammatory cytokines and growth factors of human tendon cells [4,8]. The main aim of this study was to evaluate the different response of tendon cells to different PEMF treatments and to eventually identify the more performing one, in order to translate these findings into the clinical practice. Indeed, in our opinion these *in vitro "*physical-dynamic" studies should allow to establish the best cell response together with the minimal effective PEMF dose both in terms of daily exposure and field intensity. We previously reported that a short length of exposure (4 hours) was not sufficient to trigger a functional hTC response and that the greater PEMF effects were associated with longer exposure time such as 8 and 12 hours [4]. For this reason, using these durations of exposure, in this study we compared the effects of different field intensities, length and number of exposures.

No apoptotic or necrotic events were observed after any treatment, demonstrating that PEMF are not toxic at a cellular level. hTC viability and proliferation were slightly enhanced by both 1.5mT-PEMF (single or repeated) and 3mT-PEMF treatments but with different kinetics, although a decrease was observed in R-1.5mT-PEMF. However this may be explained by a bias due to the longer culture time of these samples. As revealed by the gene expression analysis, only the 1.5mT-PEMF single treatment was able to positively influence both the tendon-specific markers and VEGF which are known to participate in the regenerative processes of tendons; in particular an increase of collagen type I/type III ratio was observed, suggesting a pro-healing effect of PEMF treatment as this ratio has been reported to be in favour of collagen type III in tendinopathy, together with other changes in total collagen composition [5,7,16,18,19,28]. In agreement with our results, other authors have already observed that, within a range of magnetic field intensity of 0.1-3.5mT (75Hz frequency), 1.5 mT gave the best results in terms of proteoglycans synthesis in cartilage explants compared to other field intensities [32].

It has been recently reported that tendon disorders, in particular in chronic tendinopathies, are characterized by an inflammatory state which involves COX2, substance P, MMPs and the infiltration of immune cells in the peritenon, [26]. However, in the physiologic tendon healing process, the inflammatory response also plays an important role, and it is orchestrated in different consequential phases. In the first phase the increase of the pro-inflammatory cytokine IL-1β induces the immunoregulatory cytokine IL-6 activation in tendons, triggering the inflammatory reaction. Once IL-1β and IL-6 have reached the peak of production, they are themselves promoters of the production of an anti-

inflammatory cytokine, IL-10, which counteracts the inflammatory reaction, thereby concluding the inflammation process [11,32]. This effect is particularly evident in our study comparing the single 1.5mT-PEMF treatment with the repeated treatment. The early increase of IL-1β, in particular after 8h exposure, indicates that 1.5mT-PEMF treatment was able to boost the early inflammatory phase responsible for the healing process. A similar effect was observed for IL-6, which lies under the direct regulatory control of IL-1β [37]; in fact, lower levels of IL-6 in R-1.5mT-PEMF treated cells was found compared to 1.5mT-PEMF ones, indicating that the timing of the response to the repeated treatment exceeded the initial inflammatory phase and entered the second, anti-inflammatory phase. A similar response has been reported in a study by Gomez-Ochoa, where a decrease of IL-1β and IL-6 and an increase of IL-10 at late time points after PEMF treatment were observed [13]. This is clearly confirmed in our study by the increase of IL-10, the recognized anti-inflammatory mediator, in parallel with the decrease of IL-1β and IL-6 at 1 and 2 days after exposure. IL-10 does not only control the "self-regulating" anti-inflammatory loop of the final phases of the inflammatory response, but is also reported to be closely connected with the tendon healing process [27] and to affect the proliferation of connective tissue cells [33]; moreover, an up-regulation of IL-10 correlates with a superior healing process compared to controls in a murine model of patellar tendon lesion [32]. Therefore the further increase of IL-10 in the repeated treatment indicates that the repetition of 1.5mT-PEMF treatment represents an additional potential advance in the healing and remodeling process. The positive effect of the 1.5mT-PEMF treatment, both as single exposure and repeated, is also confirmed by the significant increase of TGFβ, which is known to be strongly related to the tendon

healing process by stimulating the production of fibronectin by tendon cells [42]. Conversely, by increasing the field intensity, no further advantages were observed on these parameters, but rather it provoked a slightly significant increase of TNFα, an inflammatory cytokine involved in tendon degeneration [14].

The main limitation of this study is the lack of daily observations of the parameters during the R-1.5mT-PEMF treatment. This could probably explain why we did not observe a specific gene expression up-regulation in cells exposed to repeated PEMF treatments as compared to those just treated once. It has to be considered, however, that the cumulative effect exerted by repetitive exposure to electromagnetic fields is hardly detectable in gene expression since it is a short term event and is often characterized by a negative feedback mechanism. The same could be said for the cytokine release analyses, where we decided to observe the cumulative effect in cytokine production rather than the daily release. In this case, this choice allowed us to clearly identify the stronger anti-inflammatory action of the repetition of PEMF treatment, as a net effect of a higher IL-10 production, confirming the beneficial effects of similar protocols already used in clinical practice [2,20,22,44]. Furthermore, our study was performed starting from healthy tissues while cells isolated from pathological tissues could better represent the *in vivo* condition for which PEMF treatment is used, helping clarify the kinetics of inflammatory and anti-inflammatory cytokine production involved in tendon repair.

CONCLUSION

1.5mT-PEMF treatment was able to give the best results in *in vitro* healthy human tendon cell culture in term of cell proliferation, up-regulation of tendon specific-gene expression and release of anti-inflammatory cytokines and growth factors. In addition, the repetition of this low intensity treatment leads hTCs to significantly increase their IL-10 production, which is closely involved in the anti-inflammatory pathways. These "physical-dynamic" findings could be used to define the minimal effective dose both in terms of daily exposure and field intensity to be applied in pre-clinical and clinical studies. Although the clinical relevance is not direct, this investigation should be considered as the first attempt to clarify the effect of different PEMF protocols on tendon cells, in particular focusing on the potential applicability of this cell source for regenerative medicine purpose, both in surgical or in conservative treatment for tendon disorders.

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4.4 Manuscript 7: Development of an effective strategy for the tenogenic differentiation of human Adipose-, Bone Marrow-and Tendon-derived Mesenchymal Stem Cells

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Manuscript in preparation

ABSTRACT

Even though the adipogenic, chondrogenic and osteogenic differentiation potential of Mesenchymal stem cells (MSCs) have been deeply studied, the in *vitro* tenogenic differentiation of MSCs has been not well established yet. Then, the purpose of the this work is to evaluate multiple biochemical stimulations to establish the most effective treatment to promote the tenogenic differentiation of MSCs, performing also a comparison of the tenogenic potential of Bone Marrow derived Stem Cells (BMSCs), Adipose derived Stem Cells (ASCs) and Tendon stem progenitor cells (TSPCs) and select the best performing cell source. Five different combinations of growth factors and supplements (including BMP-12, bFGF, TGF-β3, IGF-1, CTGF, AA) were used as tenogenic inductive treatments. The single addition of Bone Morphogenetic Protein 12 (BMP-12) was considered

as positive control. First we selected the most effective growth factors combinations by the evaluation of the expression of two tendon markers, scleraxis (SCX) and decorin (DCN), in immunofluorescence assays. The quantification of these markers was then assessed by an immunofluorescence-based high-throughput system, Operetta® (Perkin Elmer). The gene expression of a wider range of tenogenic markers such as SCX, DCN, Mohawk homeobox protein (MKX), tenomodulin (TNMD), tenascin (TN-C) and type I collagen (COL1A1) was also evaluated. In all the cell types, BMP-12 alone increased the production of DCN, MKX and TNMD whereas the presence of Transforming Growth factors β3 (TGF β3) enhanced the production of SCX and COL1A1. In addition, we also observed that TGFβ3 actively inhibits the production of DCN and TNMD. TSPCs showed the highest levels of SCX and DCN expression with a lower donor-associated variability if compared to the other cell sources. Both BMSCs and ASCs showed to be good responders to tenogenic induction, but ASCs showed a higher predisposition to acquire the typical tenogenic phenotype. Basing on the large amount of the results obtained in this study, we can furtherly support the hypothesis that a more satisfactory tenogenic differentiation of MSCs is obtained using multiple factors rather than a single one, and that ASCs could be the best non tissue-specific cell source for cell-based tendon regeneration applications.

INTRODUCTION

Tendon disorders are a complex class of pathologies affecting a wide percentage of the population worldwide. Current conservative treatments and pharmacological therapy with non-steroidal anti-inflammatory drugs (NSAIDs)

are efficient in pain relief but fail to restore tissue homeostasis, so that in several cases the progression of the pathology eventually lead to surgical intervention. On the other side, surgical interventions are very invasive and the recovery from them is a long process and re-occurrence of the pathology is frequent (Maffulli N et al, 2006). Therefore, in the last decades the search for alternative treatment has increased and among the different possibilities Mesenchymal Stem cells (MSCs) seem to represent a valuable tool to improve the clinical outcome in case of tendon pathology. In fact, MSCs can promote the formation of a regenerative microenvironment thanks to their ability to act on resident progenitor cells and to differentiate themselves into tenocyte-like cells (Lee JY et al, 2011; Lui PP et al, 2014; Manning CN et al, 2015; Chen HS et al, 2015). However, the direct use of MSCs in the target site in *in vivo* models of tendon disorders could lead to the formation of ectopic bone or cartilage tissue (Lui PP et al, 2012; Harris MT et al, 2004). This side effect was already experienced in other MSCs application and many literature reports suggested that a priming of cells before treatment would reduce this issue. For example, the pre-differentiation of ASCs towards the osteogenic lineage resulted a successful strategy in bone repair application (Yoon E et al, 2007). Then, the previous possibility to induce MSCs toward the tenogenic lineage before using, may ameliorate the outcome avoiding an impaired tissue regeneration and remodeling. Nevertheless, differently from adipogenic, chondrogenic and osteogenic differentiation, the proper in *vitro* tenogenic differentiation protocol of MSCs is still unclear.

MSCs show dissimilar phenotypic aptitudes, depending on their specific tissue of origin. Therefore, the identification of the most suitable source of MSCs for this

application is crucial to obtain an efficient and stable differentiation toward the tenogenic lineage.

In this context, the aim of the present work is to establish an effective tenogenic treatment, able to induce the expression of a stable tenogenic phenotype in MSCs, by the assessment of the tenogenic potential of Bone Marrow Derived Stem Cell (BMSCs), Adipose derived Stem Cells (ASCs) and Tendon Stem Progenitor Cells (TSPCs) in presence of different biochemical stimuli, with the final purpose of defining the optimal protocol and cell source for regenerative medicine applications of MSCs in tendon disorders.

MATERIALS AND METHODS

MSCs isolation and culture

Waste surgical samples were collected at our Institute as under written consent of the patients (M-SPER-014.ver7 for the use of surgical waste). In particular, hBMSCs were isolated from the femoral canal of five donors who underwent to hip replacement. Bone marrow aspirates were washed in phosphate-buffered saline (PBS) and centrifuged at 623 g for 10 minutes. The fraction of mononuclear cells was suspended in complete medium (CM) composed of Dulbecco's Modified Eagle Medium High Glucose (SIGMA Aldrich), 10% fetal bovine serum (FBS, Sigma-Aldrich, St. Louis, MO, USA), 50 U/mL penicillin, 50 mg/mL streptomycin, 2 mM L-glutamine (Life Technologies) and plated in culture flasks at a density of $5x10^3$ cells/cm^2.

hASCs were isolated as previously described (de Girolamo L et al, 2013) from adipose tissue of five donors who underwent abdominoplasty. The adipose tissue was minced and then washed with PBS. The samples were digested

digestion in 0.075% type I collagenase (Worthington Biochemical Corporation, Lakewood, NJ, USA) at 37°C for 30 min. The obtained stromal vascular fraction (SVF) was centrifuged (1200 g, 10 min) and then filtered through a 100 µm nylon cell strainer (Becton, Dickinson and Company, Franklin Lakes, NJ, USA); 10^4 cells/cm^2 were plated in CM.

hTSPCs were isolated from fragments of semitendinosus and gracilis tendons collected from five donors who underwent anterior cruciate ligament (ACL) reconstruction with hamstring. After 16 hours of enzymatic digestion with collagenase type I at 0.3% w/v (Worthington Biochemical Corporation, Lakewood, NJ, USA) (Rui YF et al, 2010; de Girolamo L et al, 2013), the samples were filtered through a 100 µm cell strainer (Becton, Dickinson and Co., NJ, USA) and centrifuged (300 g, 5 minutes). hTSPC were plated at a density of $5x10^3$ cells/cm^2 in CM and maintained in incubator at 37°C in a humidified atmosphere with 5% CO_2. All the populations were cultured until passage 4, when they were detached and seeded for the following experiments.

Tenogenic induction growth factors-mediated

At passage 4, hBMSCs, hASCs and hTSPCs were seeded at seeding density of 3000 cells/cm^2 and treated with different combinations of growth factors (Table 1) for three days. Briefly, all cell types were maintained in a medium composed of HG-DMEM, 1% FBS, 1% PSG, Ascorbic Acid 25µg/ml, b-FGF 5ng/ml supplemented with one or more of the following growth factors: bone morphogenetic protein 12 (BMP-12), Connective Tissue Growth Factor (CTGF), Transforming Growth Factor β3 (TGF β3) and Insulin Growth Factor 1 (IGF-1). After three days of culture, media were changed and the cells maintained in a maintenance medium

composed of DMEM, 1%FBS, 1%PSG, AA 25µg/ml, b-FGF 5ng/ml until day 10. For each population, cells maintained in CM were considered as CTRL group.

	Medium	Growth factor
BMP-12 medium	DMEM, 1%FBS, 1%PSG, AA 25µg/ml, b-FGF 5ng/ml	BMP12 (50 ng/ml)
MIX 1		BMP12 50ng/ml CTGF 100 ng/ml IGF-1 50ng/ml TGF β3 20ng/ml
MIX 2		CTGF 100 ng/ml IGF-1 50ng/ml TGF β3 20ng/ml
MIX 3		CTGF 100 ng/ml IGF-1 50ng/ml BMP12 50ng/ml
MIX 4		CTGF 100 ng/ml BMP12 50ng/ml TGF β3 20ng/ml
MIX 5		IGF-1 50ng/ml TGF β3 20ng/ml BMP12 50ng/ml

Table 1: *the different inductive tenogenic media tested in these studies. The single addition of BMP-12 was considered as CTRL group*

Immunofluorescence analysis

Immunofluorescent staining for scleraxis (SCX, rabbit anti-human, 0.5 µg/ml) and decorin (DCN, mouse anti-human, 0.5µg/ml) was assessed in all samples. Cells were fixed with frozen methanol 100% for 5 minutes at room temperature and then washed with ice cold PBS. Samples were then treated with a blocking solution containing 1% of bovine serum albumin (BSA, Sigma Aldrich). Then, the diluted primary antibodies (for SCX, rabbit anti-human, 0.5µg/ml and DCN, mouse anti-human, 0.5µg/ml; Abcam,) were added to the samples and maintained overnight at 4°C. Then, cells were rinsed twice with PBS + 0.1% Tween 20 and then incubated with the diluted secondary antibody (Goat Anti-Rabbit IgG H&L, Alexa Fluor® 488, 2µg/ml; Rat monoclonal (SB74g) Anti-Mouse IgG2b gamma chain, Alexa Fluor® 647, 2µg/ml; Abcam) 1 hour at room temperature. Finally, the cells were washed and incubated with 0.1 µg/mL DAPI (DNA stain) for 1 min.

For an efficient quantification of these markers and for the qualitative evaluations of morphological modifications at different time points, an immunofluorescence-based high-throughput system, Operetta® (Perkin Elmer) was used at both time points.

Immunofluorescence images were captured by Operetta® (Perkin Elmer,MA, USA), and analyzed by Harmony Software. Basic Harmony software functions were used to evaluate mean fluorescence in all different channel (GFP, Far Red, DAPI), cell count (DAPI), cell dimension (phase contrast). Moreover, a protocol for identification of Decorin positive filament area in all fields was developed.

RNA extraction and gene expression analysis

A real time PCR (StepOne Plus, Life Technologies) was performed at day 3 and 10. Total RNA was extracted by PureLink® RNA Mini Kit (Life Technologies) and reverse transcripted to cDNA (5 min at 25°C, 30 min at 42°C and 5 min at 85°C) using an iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, CA, USA). Twenty ng of cDNA were used as template and were incubated with a PCR mix (50°C for 2 min, 95°C for 10 min, 40 cycles at 95°C for 15 s and 60°C for 1 min) containing TaqMan® Universal PCR Master Mix and Assays-on-Demand Gene expression probes (Life Technologies) for the following genes: SCX (Hs03054634_g1), DCN (Hs00370385_m1), COL1A1 (Hs01076777_m1), MKX (Hs00543190_m1), TN-C (Hs01115665_m1) and TNMD (Hs00943212_m1). Reactions were performed with Applied Biosystems® StepOnePlus™ (Life Technologies). The fold change in expression was normalized against the expression of the previously validated housekeeping gene YWHAZ (Hs03044281_g1). Two replicates were analyzed for each experimental group. Data were expressed according to the dCt/ddCt method.

Statistical analysis

Statistical analysis was performed by GraphPad Prism v5.0 software (GraphPad Software Inc., La Jolla, CA, USA). Data are expressed as the mean ± SD. Normal distribution of values was assayed by Kolmogorov–Smirnov normality test, while one-way analysis of variance (ANOVA) for repeated measures, with Bonferroni's correction, was used to compare data over time. *p values <0.05 were considered statistically significant.*

RESULTS

Screening study of Scleraxis and Decorin Expression

The expression of the early marker SCX and the late marker DCN was assessed by high throughput immunofluorescence experiments. Different conditions were compared to explore the effect of BMP-12, TGFβ3, CTGF, Ascorbic Acid, bFGF and IGF-I. In all the analyzed cell types, the expression of DCN was enhanced by BMP-12 alone and suppressed by TGFβ3 (Figure 1).

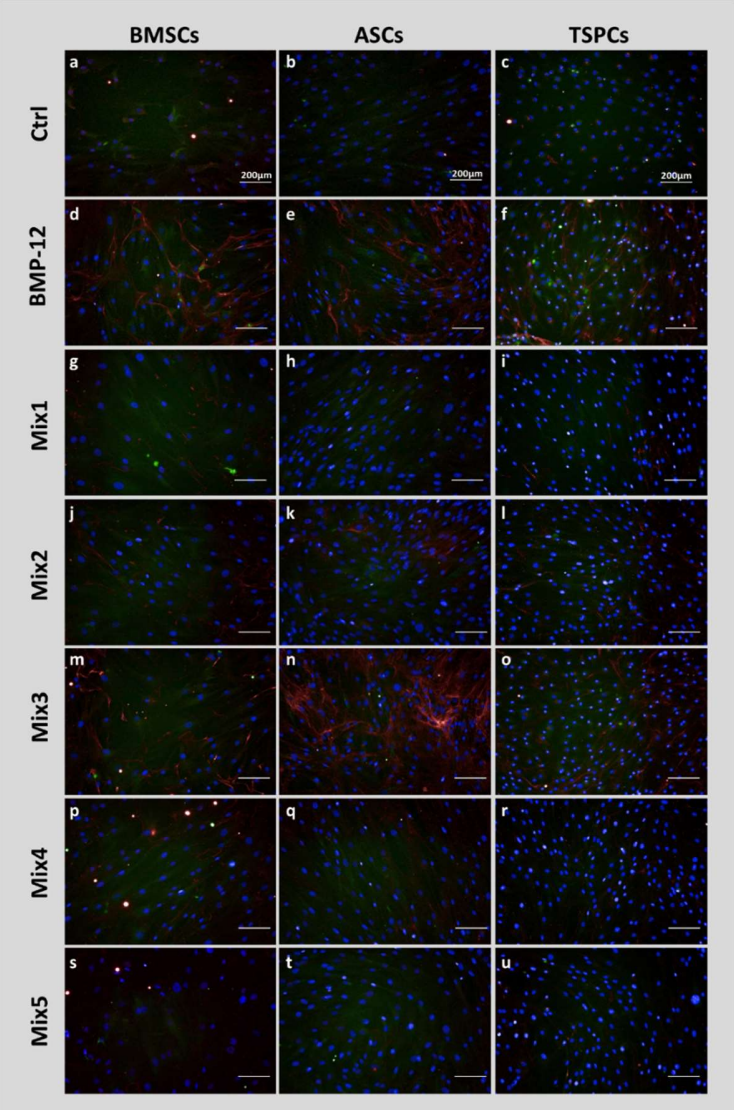

***Figure 1**: Representative micrographs of BMSCs (a-d-g-j-m-p-s), ASCs (b-e-h-k-n-q-t) and TSPCs (c-f-i-l-o-r-u) after three days of culture with different tenogenic media (Mix1, Mix2, Mix4, Mix4, Mix5). Un-treated (Ctrl, a-b-c) and BMP-12*

induced (BMP-12, d-e-f) cells for each cell types were considered as negative and positive control respectively. Magnification 10X (200µm). Immunofluorescent staining for SCX (rabbit anti-human, 0.5µg/ml; secondary antibody Goat Anti-Rabbit IgG H&L, Alexa Fluor® 488, 2µg/ml) and DCN (mouse anti-human, 0.5µg/ml; secondary antibody Rat monoclonal (SB74g) Anti-Mouse IgG2b gamma chain, Alexa Fluor® 647, 2µg/ml). DCN -- > RED SCX -- > GREEN

Indeed, all the samples treated with media containing TGFβ3 (Mix1, Mix2, Mix4 and Mix5) showed no expression of this marker after 3 days of tenogenic induction. In this setting, the expression of scleraxis did not resulted consistent within the experiments, with extremely slight differences between signal and background. At higher magnification it was possible to observe the presence of nuclear dots in BMP-12 treated ASCs, in accordance with the supposed subcellular localization of SCX (Figure S1). The lack of signal observed in Mix2 and Mix4 leaded to the exclusion of these media from further analysis.

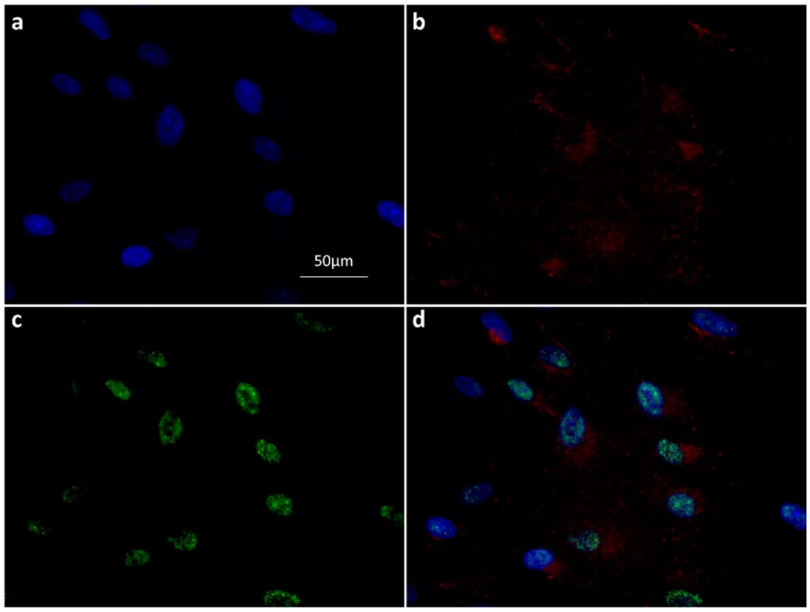

Figure S1: *Representative micrograph of ASCs treated with TGF β3 (Mix1).* ***a****-DAPI;* ***b****-DCN****; c****-SCX;* ***d****-MERGED. Magnification 40X (50µm*)

Role of bFGF and Ascorbic Acid in the induction and maintenance of DCN expression

During the induction phase (days 0-3), the presence of bFGF and AA slightly enhances the expression of DCN in media supplemented with others growth factors (BMP-12, Mix1, Mix3, and Mix5). Nevertheless, a great difference was observed in the expression of DCN when cells were maintained in culture to day 10 in DMEM+1%FBS or with the same medium with bFGF and AA. Indeed, in presence of these factors, the expression of DCN increased during the time in culture, while it was lost in non-supplemented medium (Figure 2). This observation was also confirmed in all the different cell types.

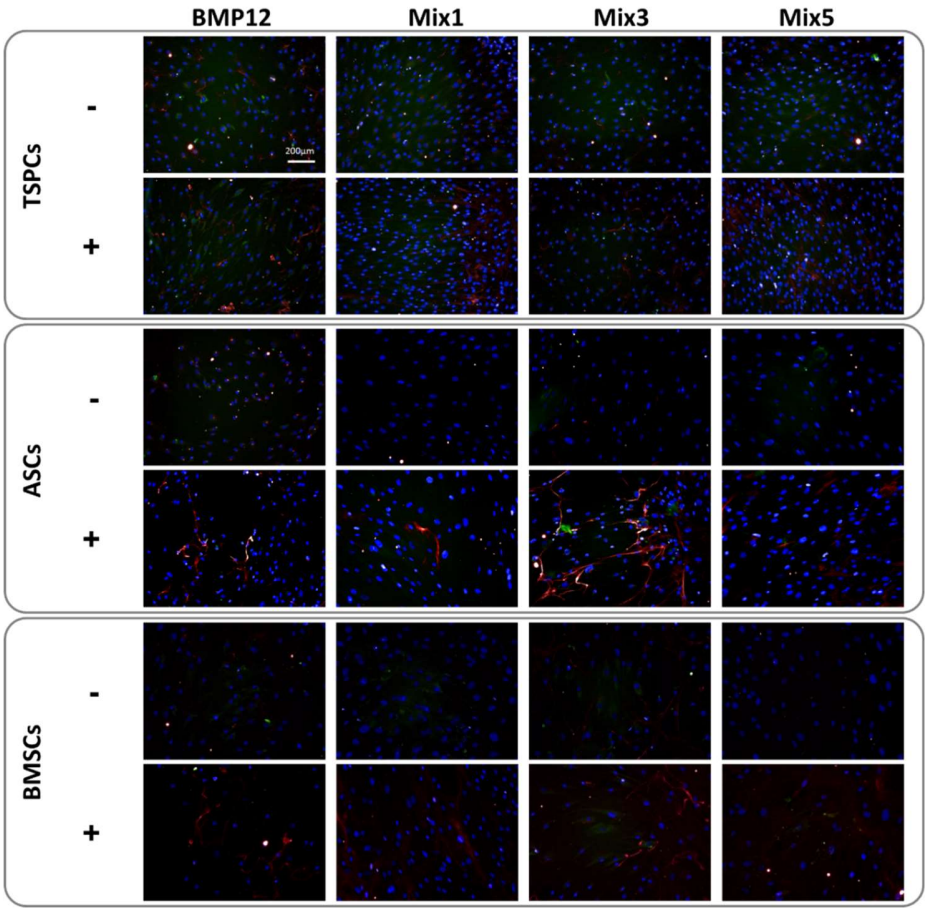

Figure 2: *Representative micrographs of differentiated TSPCs, ASCs and BMSCs without (-) and with (+) AA and b-FGF at 10 days. Magnification 10X (200µm).*

TGFβ3 induced the highest expression of SCX and COL1A1

The gene expression analysis of tendon specific markers revealed a clear role for TGFβ3 in the induction of Scleraxis and Collagen Type I, after 3 days of tenogenic

induction. Indeed, while BMP-12 alone was able to upregulate slightly these markers, media containing TGFβ3 (Mix1 and Mix5) enhance their expression, in particular in TSPCs and ASCs. The inhibitory effect of TGFβ3 on DCN expression was confirmed by the gene expression analyses. The expression of the early tenogenic marker MKX was also enhanced by TGFβ3, but only in TSPCs cells, while all other media and cell types demonstrated lack of induction of this transcription factor (Figure 3).

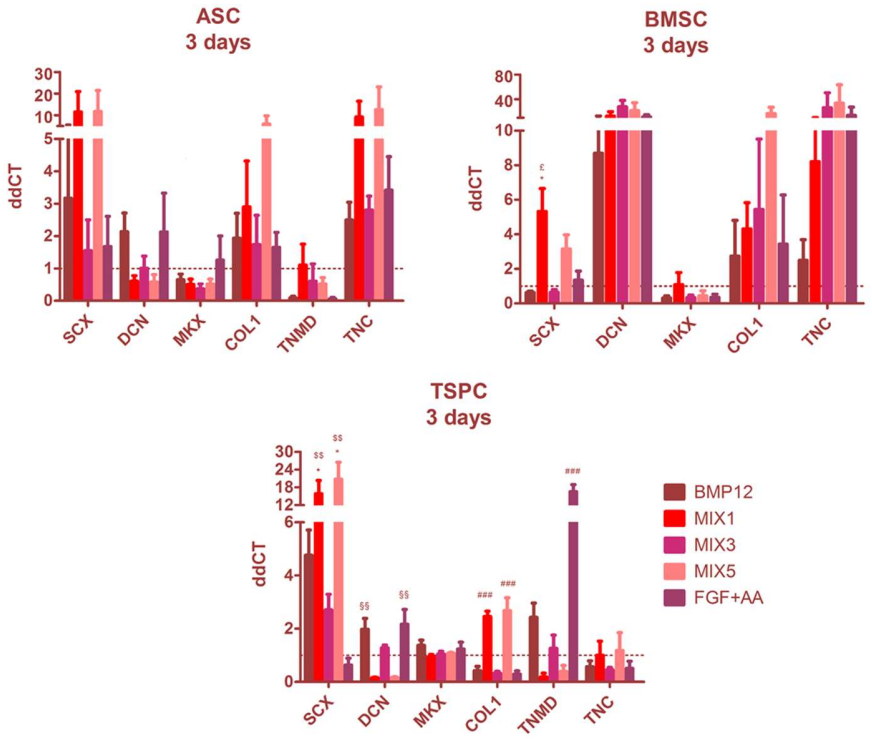

Figure 3: *Gene expression analysis of SCX, DCN, MKX, COL1, TNMD, TN-C. Data are expressed as ddCT; vs CTRL: * p<0.05; vs BMP12: $^{£}$ p<0.05; vs all: $^{###}$ p<0.001; vs MIX1 and MIX5: §p<0.01; vs FGF+AA: $^{$$}$p<0.01*

At 10 days, and in specific one week after the shift from induction to maintenance in bFGF+AA supplemented medium with 1%FBS, the expression of SCX and COL1A1 was found to be still upregulated in cells treated with TGFβ3 containing media (Mix1 and Mix5). Interestingly, the production of DCN resulted increased in the same media, especially for TSPCs, in contrast with what observed after 3 days of induction. In TSPCs the expression of MKX is significantly increased.

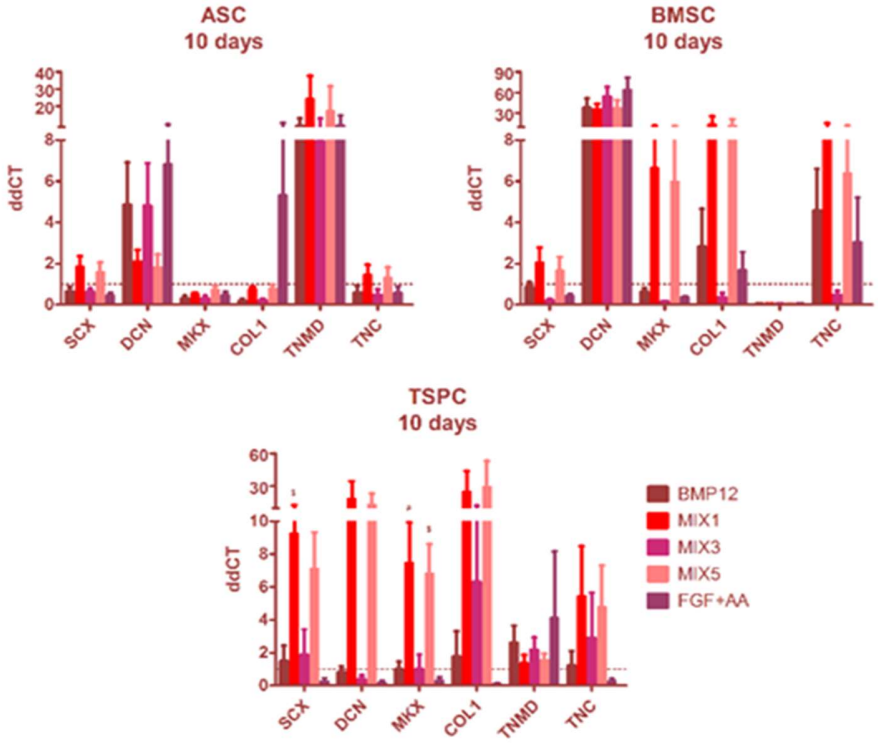

Figure 4: *Gene expression analysis of SCX, DCN, MKX, COL1, TNMD, TN-C. Data are expressed as ddCT; vs all: # p<0.05; vs FGF+AA: $ p<0.05; $$ p<0.01*

TSPCs is the most suitable cell type for tendon differentiation and ASCs performed better than BMSCs

In terms of tendon markers expression and morphology, TSPCs appear as the best performing cell type in all of our experiments. Indeed, basal expression of tendon specific transcription factors (MKX and SCX) were higher with respect to ASCs and BMSCs. Moreover, BMP-12 alone was able to exert a prompt effect on these cells, while other growth factors were required by MSCs. Nevertheless, ASCs were able to express large amount of COL1A1 and DCN, even compared to TSCPs, and for what concern the expression of SCX, MKX and TNMD they provided higher level of expression with respect to BMSCs (Figure 5).

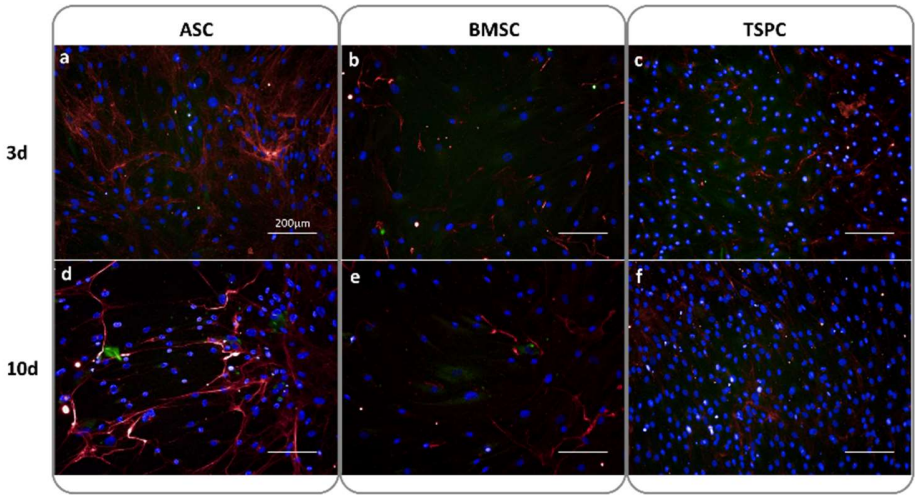

Figure 5: *Representative micrographs of ASCs (a-d), BMSCs (b-e) and TSPCs (c-f) treated with Mix3, at 3 and 10 days. Magnification 10X (200µm)*

DISCUSSION

The main finding of our work is that a proper *in vitro* tendon differentiation protocol should take in account different stages, from cell commitment to

tenocyte maturation. Our proposed tenogenic differentiation protocol comprises a first phase of 3 days induction with BMP-12, TGFβ3, bFGF, Ascorbic Acid and IGF-I in presence of 1%FBS, followed by a second phase of at least 7 days where cell should be maintained in 1% FBS, ascorbic acid and bFGF, at concentration indicated in Materials and Methods section.

In the first phases the increase in tendon specific transcription factor is enhanced by TGFβ3 and BMP-12, while a second phase characterized by matrix deposition and fibres maturation then follows. This second phase appears to be induced by ascorbic acid under the guidance of previous inductive signals. Our observation are in accordance with the physiological tenogenic differentiation cascade (Dex S et al, 2016).

Transforming Growth Factor β superfamily (TGFβ) has been extensively investigated in several literature reports and up to today together with TGFβ, Bone Morphogenetic Proteins (BMPs) and Growth Differentiation Factors (GDFs) represent the most promising molecular signals driving the tenogenic differentiation of MSCs (Havis E et al, 2014; Park A et al, 2010; Lee YJ et al, 2011; Shen H et al, 2013; Stanco D et al, 2015; Barsby T et al, 2014).

Other growth factors such as Connective Tissue Growth Factor (CTGF), Insulin Growth Factor 1 (IGF-1), basic Fibroblast Growth Factor (b-FGF), that exert a more incisive role in the tendon healing rather than in tendon development, have been investigated and their involvement in cell differentiation, proliferation, chemotaxis and ECM synthesis have been reported in literature (Ackermann P et al, 2013; Muller SA et al, 2015; Lu YF et al, 2016). It has been demonstrated that b-FGF is associated with the maturation of tenocytes (Chan J et al, 1998) and that it is able to stimulate collagen type I and Type III production

(Chan BP et al, 2000), whereas CTGF increases the expression of collagen type I and tenascin-C in MSCs (Lee CH et al, 2010). IGF-1 is particularly involved in the remodeling stages of the healing process and it is able to increase the levels of mRNA for collagen, tenomodulin and scleraxis (Herchenhan A et al, 2014). It is also currently known that the addition of ascorbic acid promotes the synthesis of collagen, the maintenance of the ECM in tendon and ligament tissues, and therefore it facilitates the healing process of the tissue (Lui PP et al, 2016; Rehmann MS et al, 2016).

It is evident that the stimulation with TGFβ3 induced the expression of specific transcription factors in tendon derived progenitors, in particular of SCX that appears to be in the cascade right after tendon progenitor stage and before tenoblast phase, as well as it happens for the expression of collagen (Brent AE et al, 2003; Havis E et al 2014). After this phase, the tendon differentiation process should move forward up to the proper tenocyte, that assumes a longer morphology and express later tendon markers, such as TNMD and DCX (Dex S et al, 2016), an event that follows the treatment by ascorbic acid and bFGF, as also showed by our findings.

Since SCX represent the most recognized marker for tendon lineage commitment (Schweitzer R et al, 2001; Shukunami C et al, 2006), our first goal was to identify the best treatment to induce its expression. In fact, SCX is a bHLH transcription factor expressed in tendon progenitor cells in the mouse embryo (Schweitzer R et al, 2001), and its ectopic expression could drive human BMSCs to express markers of tendon progenitors (Alberton P et al, 2012).

Our results clearly show that this was obtained only when cells were cultured in presence of TGFβ3. The influence of TGFβ in tendon development was described

in 2009 by Pryce and colleagues (Pryce BA et al, 2009) and the potential of this mediator in SCX induction was then verified in equine embryonic stem cells (Barsby T et al, 2013). Nevertheless, it is also known that TGFβ superfamily induce a wide number of target genes, and it is clearly involved in bone formation (Wu M et al, 2016; Labour MN et al, 2016; Ripamonti U et al; 2016). Thus, its solely application to MSCs would not allow for a precise tendon lineage induction. In our experiments, TGFβ was applied in combination with other growth factors, in particular BMP-12, that is described in literature as the main trigger of tendon differentiation. Indeed, BMP-12 is able to induce tendon specific markers in ectopic rat tissue, with a relatively low expression of bone and cartilage markers (Berasi SP et al, 2011), and many papers confirm its central role in tenogenic induction in both *in vitro* and *in vivo* scenario (Heisterbach PE et al, 2012; Lou J et al, 2001; Majewski M et al, 2008; Lee JY et al, 2011). Nevertheless, it was described as not sufficient for the induction of a proper tendon-like phenotype (Stanco D et al, 2015), but its use in combination with TGFβ3 could represent a strong and specific tenogenic inductive signal. In our experiments, BMP-12 alone showed a prompt increase of DCN expression, that could led to a reduced tendon repair ability and matrix deposition (Hildebrand A et al, 1994; Danielson KG et al, 1997). Thus, the possibility to avoid these events by the addition of TGFβ3 supports the use of both the growth factors in the first phases of tenogenic induction.

Moreover, the physiological expression of TGFβ was reported in adult tendon after injury (Chan KM et al, 2008, Fenwick SA 2001), as well as SCX but it is also correlated with hypertrophic scar tissue formation and fibrosis (Lian et al, 2016) suggesting caution in its possible clinical application. However, among the

different isoforms, TGFβ3 possesses anti-scarring properties and has proven to be able to contrast the TGFβ1 and TGFβ2 action of inflammatory cells activation and abnormal connective tissue formation (Durani P et al, 2008).

Beside SCX induction, we observed that until its removal from culture medium, TGFβ3 inhibits the production of DCN mRNA and protein. DCN is a small leucine rich proteoglycan (SLRP) protein that possess both structural and signaling physiological functions, as it is involved in Collagen type I fibrils formation and mechanical properties (Reese SP et al, 2013; Kalamajski S et al, 1998; Orgel JP et al, 2009; Pogany G et al, 1992;), but it is also active in angiogenesis modulation (Järveläinen H et al, 2015). In particular, it has been suggested that this action depends on the ability of DCN to neutralize TGFβ action by direct binding (Yamaguchi Y et al, 2016; Border WA et al, 1992; Hildebrand A et al, 1994; Schönherr E et al, 1998). Despite the bi-unique inhibitory interaction between TGFβ and DCN, the real effects and cause of this process are still unclear and further studies are needed to clarify this mechanism. For what concern the structural role of DCN, its ability in binding collagen fibers could prevent the deposition of abnormal extracellular matrix resulting in an inhibitory effect on the possible development of fibrotic tissue (Hildebrand A et al, 1994). Then, the ability to induce DCN in tendon differentiated cells represents an encouraging aspect for the achievement of a physiological matrix production. BMP-12 showed to play a role in the induction of markers of tendon matrix maturation, but extremely higher results were obtained with ascorbic acid at both 3 and 10 days of differentiation protocols. Indeed, ascorbic acid was described as able to induce extracellular matrix deposition in a number of different approaches (Rehmann MS et al, 2016; Chen X et al, 2009), and its ability to induce Collagen

type I (Takeuchi Y et al, 1993; Hering TM et al, 1994) is clearly coupled with the increased expression of DCN as resulting from our experiments. Nevertheless, ascorbic acid is not able to induce SCX, indicating that it would not be sufficient for tenogenic differentiation, but the ability in reducing collagen type II and osteocalcin expression (Ni M et al, 2013) make ascorbic acid an optimal effector of matrix deposition and maturation phases during tendon differentiation, as well as of tendon phenotype maintenance. At least for what concern TSPCs, it also showed the ability to increase TNMD expression, confirming its important role in the late stages of tendon development.

Another marker of tendon matrix is TN-C. It was used in the first years of tendon differentiation era (Lee et al, 2005), before the discovery of SCX (Chiquet M et al, 1984; Kardon G et al, 1998; Huang AH et al, 2015), and even if it is not as specific as other components of the extracellular matrix (Makie EJ et al, 1996), this glycoprotein represents an early marker of matrix (Huang AH et al, 2015). Indeed, the TNC expression pattern in our experiments was similar to the SCX and COL1 ones, since it is upregulated in presence of TGFβ3, confirming that this stimulation is able to enhance an early response towards tenogenic lineage.

Differently, a very early tendon marker such as MKX that is a transcription factor to SCX (Otabe K et al, 2015; Nakamichi R et al, 2016), was just slightly induced by our treatments and only in TSPCs, while no induction was observed after treatment of ASCs and BMSCs. Since its effect on tenogenic induction appear to be stimulated through TGFβ pathway (Liu J et al, 2015), it is possible that our direct induction of SCX by TGFβ bypassed the MKX involvement, reaching a more differentiated stage without the action of this particular transcription factor.

CTGF is another important growth factor for the induction of tendon matrix and specific transcription factor production (Ni M et al, 2013). Nevertheless, according to our results it was not able to induce any further increase in the expression of SCX, COL1 and TNC when used in combination with TGFβ3, BMP-12 and IGF-I, at least for what concern the treatment of ASCs and TSPCs. In fact, it showed a slight enhancement of the expression these markers in BMSCs, suggesting a role for CTGF in this particular cell type, as already suggested by other authors (Lee CH et al, 2006; Sassoon AA et al, 2012).

The differences among the analyzed cell types underlined the different potential of each cell source for tendon regeneration approaches. In particular, as widely accepted, TSPCs represent the gold standard for this particular application (Bi Y et al, 2007, Lui PP et al, 2011, Stanco D et al, 2015). The basal expression of the main specific markers, such as SCX, MKX and TNC was indeed higher in this population with respect to adipose and bone marrow derived counterparts, and these gaps were increased by tenogenic induction. Nevertheless, the low availability of TSPCs (Lui PP et al, 2011) and the rapid de-differentiation that occurs *in vitro* (Tan Q et al, 2012) suggested that other sources of MSCs could be a better choice for the clinical translation of this approach. Indeed, both ASCs and BMSCs showed potential in tenogenic differentiation but according to our data ASCs are more prone to produce tendon like extracellular matrix, while BMSCs are more easily induced in early stage of tendon differentiation. Indeed, it is known that adipose tissue derived cells spontaneously produce COL1 and TNMD (Stanco D et al, 2015; Saiki A et al, 2009), and they were able to increase also SCX, DCN and TNC expression after stimulation with BMP-12 and TGFβ3. Nevertheless, the ability to respond to these kind of stimuli, was accompanied

by concerns about the induction of cartilage markers such as COMP and aggrecan (Park A et al, 2010; Martinello T et al, 2012), and then the possible formation of ectopic cartilage. On the other hand, BMSCs showed a higher basal expression of SCX with respect to ASCs but at the same time, lower levels of all the other matrix markers. After stimulation with BMP-12, TGFβ3 and in particular CTGF, the increase in COL1 and TNC expression indicated a good response of this cell type to early induction of tendon differentiation, while the later phase of matrix production and maturation was not induced as demonstrated by the lack of DCN and TNMD overexpression after treatments. Most of the previous works support these observations, even if TNMD expression was reported after BMSCs tenogenic induction by different methods (Leung M et al, 2013; Schneider PRA et al, 2011; Luo Q et al, 2009). Different experimental conditions and TGFβ-isoform specific effects could explain these apparent inconsistencies, while confirming our findings for what concern SCX, COL1 and TNC expression. Moreover, some concerns are present for possible ectopic tissue formation after tenogenic induction of BMSCs, in particular muscle and bone (Hankemeier S et al, 2005; Pietschmann M et al, 2013; Bi Y et al, 2007; Harris MT et al, 2004), while cartilage transdifferentiation did not emerged as a possible side effect (Tan SL et al, 2012). Taken together, all of these informations suggest that ASCs would produce a more tendon-like extracellular matrix with respect to BMSCs, and could be preferred for tendon regenerative applications.

A limitation of the present study is represented by the lack of bone and cartilage specific markers assessment during differentiation. Nevertheless, the induction of SCX was described as alternatively with respect to SOX9 (Sugimoto Y et al, 2013), thus suggesting that cartilage differentiation should be prevented by

treatments inducing this tendon specific marker. Another limitation is represented by the lack of an efficient antibody for immunofluorescence application to SCX, resulting in a high background signal. Different previous works relied on this method for SCX investigation, but they often showed its expression in the cytoplasm, while the localization of SCX should probably be nuclear. The absence of mechanical induction of tendon differentiation in our experiments could have also produced an underestimation of tenogenic potential of our protocol and cell types, since the mechanical stretch was already described as an efficient method to enhance tendon specific markers expression and tenocyte-like morphologic appearance (Li Y et al, 2015).

In the future, a wider investigation over the effect of IGF-I, as well as the application of our protocols to 3D culture and the use of mechanical and biophysical stimulation, will be taken in account to increase tenogenic inductive effectiveness and to advance with the knowledge in this field. In particular, Pulsed Electromagnetic Fields will be applied in the same experimental environment, since they already demonstrated SCX-inductive ability (de Girolamo L et al, 2013; de Girolamo L et al, 2015).

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5. Development of an animal model of tendinopathy to test the efficacy of the biophysical stimulation of endogenous stem/progenitors cells in tissue healing (*Aim3*)

Another important aim of this PhD research is represented by the demonstration of the possibility to use MSCs as therapeutic target in degenerative pathologies. Indeed, the promising results obtained by biophysical stimulations of TSPCs in terms of biological response opened the field for the development of a non-invasive enhancement of tissue healing, targeting the regenerative strategies on resident MSCs. To investigate the efficacy of this "resident cell-based" therapy in tendinopathy, first an animal model of chemically-induced tendinopathy was developed by collagenase injection in the Achilles tendon of male Sprague-Dawley rats (Manuscript 8: Perucca Orfei C et al., 2016, published in Plos One). Although the collagenase induced tendinopathy is a recognized model to study this condition, the lack of consensus in term of collagenase dose and uncomplete characterization of the pathological progression over time has led to the need of the establishment of a proper

pathological model. An *in vivo* study of PEMFs effect on tendon healing was then performed in the pathological model developed in the first study. (Manuscript 9: ongoing study).

5.1 Manuscript 8: Dose-related and Time-dependent Development of Collagenase-induced Tendinopathy in Rats

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ABSTRACT

Tendinopathy is a big burden in clinics and it represents 45% of musculoskeletal lesions. Despite the relevant social impact, both pathogenesis and development of the tendinopathy are still under-investigated, thus limiting the therapeutic advancement in this field. The purpose of this study was to evaluate the dose-dependent and time-related tissue-level changes occurring in a collagenase-induced tendinopathy in rat Achilles tendons, in order to establish a standardized model for future pre-clinical studies. With this purpose, 40 Sprague Dawley rats were randomly divided into two groups, treated by injecting collagenase type I within the Achilles tendon at 1 mg/mL (low dose) or 3 mg/mL (high dose). Tendon explants were histologically evaluated at 3, 7, 15, 30 and 45 days. Our results revealed that both the collagenase doses induced a disorganization of collagen fibers and increased the number of rounded resident cells. In particular, the high dose treatment determined a greater neovascularization and fatty degeneration

with respect to the lower dose. These changes were found to be time-dependent and to resemble the features of human tendinopathy. Indeed, in our series, the acute phase occurred from day 3 to day 15, and then progressed towards the proliferative phase from day 30 to day 45 displaying a degenerative appearance associated with a very precocious and mild remodeling process. The model represents a good balance between similarity with histological features of human tendinopathy and feasibility, in terms of tendon size to create lesions and costs when compared to other animal models. Moreover, this model could contribute to improve the knowledge in this field, and it could be useful to properly design further pre-clinical studies to test innovative treatments for tendinopathy.

INTRODUCTION

Tendinopathy is a big burden in clinics and it represents 45% of musculoskeletal lesions [1]. In particular, athletes and middle-aged people are frequently affected by tendinopathy of Achilles, patellar and supraspinatus tendons. The severity of tendon injuries ranges from transient pain and inflammation to chronic conditions leading to tears or total tendon ruptures [1, 2]. The poor healing capability of damaged tendons is related to their scarce blood supply and the compromised metabolic activity of resident cells [3] that determine an impaired tissue homeostasis [4]. The histopathological appearance of injured tendons shows collagen degeneration and fiber disorganization, increased vascularization and increased presence of resident cells, tissue metaplasia, and occasionally formation of fatty and bony deposits [5, 6]. However, despite its clinical significance, the pathogenesis and development of the tendinopathy are still

under-investigated, thus limiting the therapeutic progress in this field. In fact, the current conservative treatments are still mainly symptomatic, whereas surgical approaches have a poor success rate and require a long recovery time [7].

In this contest, a better knowledge of tendinopathy progression throughout its phases could be reached through the development of an efficient *in vivo* model focused on the choice of the most effective dose of collagenase at an exact temporal window to induce the acute phase of the disease.

Despite an ideal animal model able to reproduce all aspects of human tendinopathy has not been identified so far, rat represents the most popular species to model the Achilles tendinopathy, thanks to its suitable size for surgical approaches and tissue retrieval, and its easy handling. Moreover, the rat model of Achilles tendinopathy has been extensively used in preclinical research, because of the similar conditions [8, 9] and the genetic homology to humans [10]. The most common techniques to develop rodent models of tendinopathy are based on mechanical overuse or chemical factors, such as collagenase, corticosteroids, cytokines (TGF-β1) and substance P [8, 9]. However, the mechanical overuse model is not completely accepted due to its scarce reproducibility and to the role of inflammation that does not equate to tendinopathy [8, 11]. Among the chemical-induced tendinopathy models, it has been shown that collagenase type I can provoke collagen fiber disruption and changes in biochemical and biomechanical features of the tendon, better resembling the main histopathological characteristics and functional impairments of human tendinopathy [8, 12, 13]. Thus, this injection model can represent a valid approach to induce and study the development of this

pathology [14]. However, despite collagenase seems to be the most interesting agent to generate a consistent and reproducible model of tendinopathy, to date, standardized protocols have not been defined yet. Indeed, there is no agreement on which is the concentration, volume and site of injection, and time of occurrence of collagenase-induced lesions [13, 15-20].

The purpose of this study was to evaluate the cellular and tissue-level changes occurring in a collagenase-induced Achilles tendinopathy in rats at different time points by using two collagenase concentrations. In particular, we want to accurately investigate the development of the disease throughout its phases, in order to establish a standardized model for future pre-clinical studies that resembles as closely as possible the human pathology.

MATERIALS AND METHODS

Ethics Statement

The Mario Negri Institute for Pharmacological Research (IRFMN) Animal Care and Use Committee (IACUC) approved the study (Permit N. 06/2014-PR). The IRFMN adheres to the principles set out in the following laws, regulations, and policies governing the care and use of laboratory animals: Italian Governing Law (D.lgs 26/2014; Authorization n.19/2008-A issued March 6, 2008 by Ministry of Health); Mario Negri Institutional Regulations and Policies providing internal authorization for persons conducting animal experiments (Quality Management System Certificate – UNI EN ISO 9001:2008 – Reg. N° 6121); the NIH Guide for the Care and Use of Laboratory Animals (2011 edition) and EU directives and guidelines (EEC Council Directive 2010/63/UE). The Statement of Compliance (Assurance) with the Public Health Service (PHS) Policy on Human Care and Use

of Laboratory Animals has been recently reviewed (9/9/2014) and will expire on September 30, 2019 (Animal Welfare Assurance #A5023-01). The animals were regularly checked by a certified veterinarian responsible for health monitoring, animal welfare supervision, experimental protocols and procedure revision. All surgeries were performed under general anesthesia, and all efforts were made to minimize suffering.

Study design

Forty 12-weeks-old male Sprague Dawley rats (Rattus norvegicus) (Envigo, Huntingdon, UK) (mean body weight 347 ± 9 g) were used in this study. In the absence of suitable data to perform the power analysis, the sample size was calculated according to the Mead's resource equation (E=N-T, 10<E<20). Thus, considering two treated limbs per animal for a total of 16 treated tendons per time-point, the sample size was calculated as follows: E=(16-1)-(4-1)=12. The rats were randomly divided into two treated groups that were injected within the Achilles tendon with collagenase type I (collagenase from Clostridium histolyticum, Worthington Lakewood, NJ, USA, 185 IU/mg): 1) collagenase 1 mg/mL defined as low dose (LD); 2) collagenase 3 mg/mL defined as high dose (HD). In each group, the contralateral tendon was treated either with phosphate buffered saline (PBS) and served as control (CTRL), or left untreated and served the sham control (S-CTRL). For each time point, 4 tendons were injected with LD, 4 tendons with HD, 4 tendons received PBS and 4 tendons were left untreated (n=4). Tendon explants were analyzed at 3, 7, 15, 30 and 45 days.

***In vivo* procedures**

The rats were anesthetized via inhalation of isoflurane (3%; Merial, Duluth, Georgia, USA) and maintained with an intraperitoneal injection of ketamine

hydrochloride (80mg/kg; Imalgene, Merial, Milan, Italy) and medetomidine hydrochloride (1mg/kg; Domitor, Pfizer, New York City, NY, USA). All animals also received a preoperative intramuscular single injection of cefazolin (30mg/kg; Cefamezin, Teva, Petah Tikva, Israel). Using aseptic technique, after shaving and disinfection, a longitudinal 0.5 cm skin incision was performed through a medial approach to expose by blunt dissection the central region of the Achilles tendon. Then, all animals were injected with LD or HD of type I collagenase dissolved in 30 µL of PBS by means of a 30G needle into the central portion of the tendon, and with PBS into the contralateral tendon as controls (Fig 1). Finally, the skin was sutured by separated stitches with Prolene 4/0 (Johnson & Johnson, New Brunswick, NJ, USA). Atipamezole (1mg/kg; Antisedan, Pfizer, New York City, NY, USA) was administered subcutaneously to recover the animals from general anesthesia. After 3, 7, 15, 30 and 45 days, the rats were euthanized by CO2 inhalation to harvest the Achilles tendons for the histological investigations.

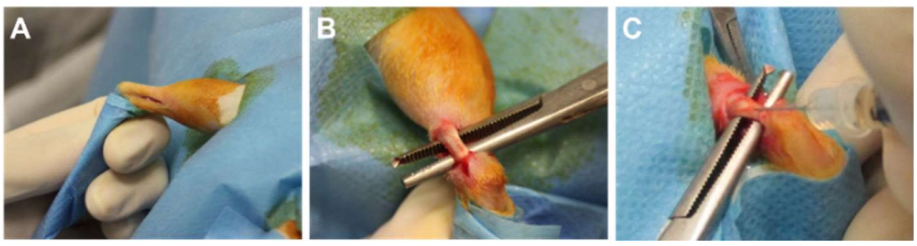


Figure 1. *Surgical approach. A) Longitudinal incision of the skin in the medial portion of the hind limb. B) Achilles tendon exposure by blunt dissection. C) Injection of collagenase type I within the Achilles tendon structure.*

Histological analysis

Tendon specimens were fixed in 10% formalin for 24h. Then, they were dehydrated in alcohol scale before embedding in paraffin and cutting into 5 µm longitudinal sections. The slides were stained with haematoxylin and eosin (H&E) to evaluate the tendon morphology of collagenase-treated groups compared to tendons treated with PBS (CTRL) or untreated native tendons (S-CTRL). Photomicrographs of the tissue were captured through an Olympus IX71 light microscope and an Olympus XC10 camera (Japan). Four sections of each sample were randomly selected and evaluated by two blinded observers to assess the tendon morphology according to a modified semi-quantitative grading score from 0 to 3 proposed by others [21, 22] (see S1 Table). The score analyzed the fiber structure and arrangement, resident cell density and appearance, infiltration of inflammatory cells, neovascularization and fatty deposits. According to this grading system, a perfectly normal tendon obtained score 0, whereas a score of 18 was assigned to maximally abnormal tendons.

Statistical analysis

Comparisons between groups and time points were analyzed by the Mann-Whitney U test (GraphPad Prism v5.00 Software, La Jolla, CA, USA). All data are expressed as means ± standard deviation (SD). Values of $p<0.05$ were considered statistically significant. The interrater reliability of the examiners' scores for each technique was calculated with intraclass correlation coefficient (ICC): ICC = 1, perfect reliability; ICC > 0.75, excellent reliability [23].

RESULTS

Histopathological findings

The S-CTRL tendons showed a uniform appearance of compact, well-aligned collagen fibers with a normal presence of spindle-shaped tenocytes disposed parallel to the fiber pattern, and, as expected, no degenerative events were observed during the whole study period (Fig 2).

Differently, at day 3, the CTRL group exhibited a loss of fiber organization that also appeared partially fragmented along the route of the needle injection (Fig 3A). However, a complete recovery of the tendon structure was spontaneously achieved starting from day 7 (Fig 3D) and maintained until the last time point (day 45; Fig 3A).

The LD-treated tendons showed a mild degeneration throughout the time points. At day 3, the LD-treated tendons showed an abnormal presence of fatty deposits associated with the loss of fiber organization (Fig 3B, black arrows) disseminated with an increased number of slightly rounded resident cells (Fig 3B, white arrow). At later time points, the presence of a high number of rounded cells was the most distinctive feature of degeneration in these samples, by the way decreasing progressively until day 45 (Fig 3E, H, K, N).

Three days after the collagenase injection, the HD-treated tendons exhibited a moderate to marked collagen matrix disorganization with a great increase of cell density consisting mostly in rounded cells (Fig 3C). Moreover, a marked neoangiogenesis associated with the presence of several inflammatory cells was found (Fig 3C, white arrow). At days 7 and 15, the HD-treated tendons showed a complete fiber disorganization, in which the pattern was no more identifiable and the increased resident cells showed a rounded morphology (Fig 3F, I).

Particularly on day 7, a marked increase in vascularity was detected related to the presence of several inflammatory cells (Fig 3F, asterisk). Furthermore, at day 15, a substantial presence of lipid vacuoles was found (Fig 3I, black arrow). Thirty days after the HD collagenase injection, the collagen fibers appeared disseminated of rounded and proliferative cells towards a new connective tissue deposed within the disrupted collagen fibers (Fig 3L). From day 30 to day 45, the collagen structure was resized and reshaped in a parallel organization, and a decreased cell number was detected at 45 days (Fig 3O).

In general, comparing the two treated groups, the HD collagenase induced a progressive degeneration of the tendon tissue, with a peak around day 15, while the injection of LD collagenase exerted its effect earlier (day 3) with no further increase of the pathological aspects during the following time points. Moreover, the LD treatment produced a mild degeneration with respect to the HD, particularly in terms of angiogenesis, fatty infiltration and fiber disorganization. Indeed, even if both treatments led to a spontaneous regeneration of the tissue at day 45, the effect of the treatment was still evident in the HD group, while the LD-treated tendons appeared similar to the CTRL (Fig 3N, O).

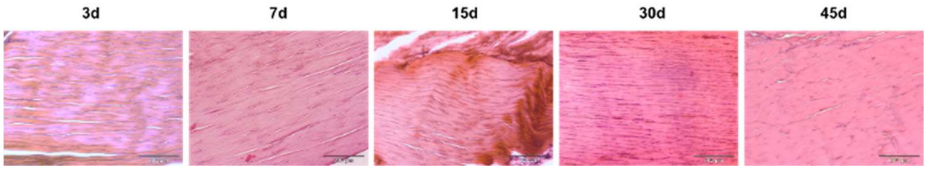


Figure 2. *Histologic appearance of S-CTRL tendons at different time points. Representative micrographs of the histopathological analysis; H&E staining. Scale bars 200 µm (10X).*

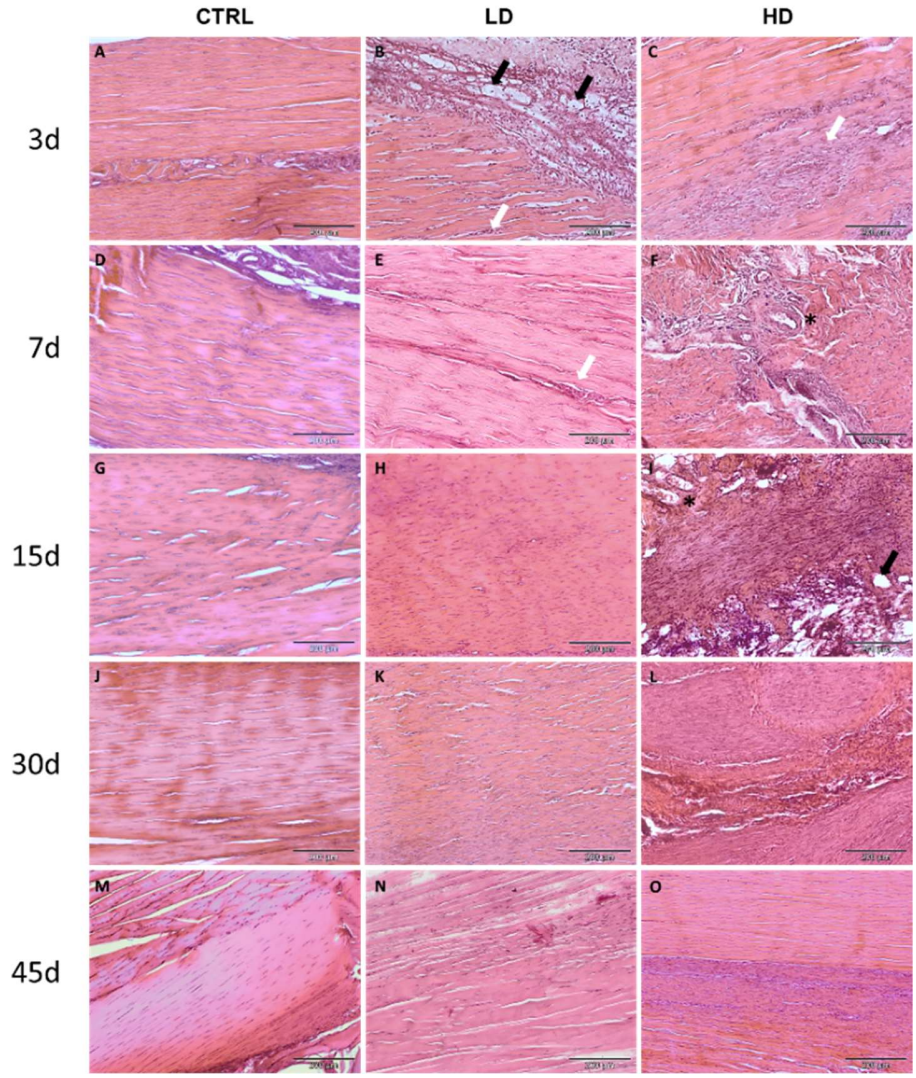


Figure 3. *Histological appearance of CTRL, LD and HD-treated tendons at different time points. Representative micrographs of the histopathological*

*analysis, H&E staining. Black arrows indicate fatty deposits; White Arrows: representative zone with high cellularity; *: vessels. Scale bars 200 µm (10X).*

Histopathological score analysis

The interrater reliability of the scoring analysis performed by two blinded examiners was good (ICC 0.73). The total and specific histopathological scores are presented in the histograms in Fig 4A.

The injection of collagenase induced deep changes in the histological appearance of the treated tendons resulting in a significantly worse total score of the HD and LD groups compared to the CTRL group at all time-points (with the exception of LD at day 15; Fig 4A). The HD group reached the worst total score at day 15 (16.5±2.1), while the LD one reached a maximum of 9.7±0.4 at day 3. Remarkably, the total score in the HD group decrease significantly between day 3 and 45 ($p<0.05$). Both concentrations of collagenase produced a visible damage on fiber arrangement, though significant differences were just observed at day 15 in the HD group respect to CTRL ($p<0.05$) and to LD ($p<0.05$). Analyzing the temporal changes in terms of disorganization of collagen fibers, we found a progressive score worsening at least up to 15 days (1.75±0.4 at day 3, 3±0.0 at day 15, $p<0.05$), but then a subsequent spontaneous regeneration was observed (1.17±0.3 at day 45, $p<0.05$) (Fig 4B).

At day 3, cell density was increased by all the treatments with a significant increase between CTRL and HD ($p<0.05$) or LD groups ($p<0.05$) (Fig 4C). The cell density of the HD group was higher at all time-points respect to CTRL and LD treatments, with a significant increase respect to LD at day 15 ($p<0.05$). Cell morphology was also affected by the treatments. In fact, a higher number of

rounded cells was observed in the LD and HD groups with respect to CTRL at all time-points (Fig 4D). In particular, at day 7, both LD and HD group showed a higher score with respect to CTRL ($p<0.05$). Moreover, the HD group showed significant increases also at day 15 and 45 compared to CTRL ($p<0.05$). Nevertheless, in the HD group, a progressive decrease of rounded cells was observed at day 45 with respect to day 3, 7 and 15 ($p<0.05$).

The infiltration of inflammatory cells was observed in all samples at day 3. By day 7, they almost disappeared in CTRL group, while they were persistent in LD and HD ones. In particular, in the LD group, a significant increase was observed with respect to CTRL at day 7 and day 30 ($p<0.05$), whereas in the HD group it was found only at day 15 ($p<0.05$) (Fig 4E).

The presence of new vessels was evident in all collagenase-treated tendons (Fig 4F), specifically, their number significantly increased in both LD and HD groups with respect to CTRL up to 15 days ($p<0.05$). In particular, HD groups showed a greater presence of vessels compared to LD group at day 15 ($p<0.05$). A similar behavior was observed in terms of fatty deposits (Fig 4G). In fact, at day 3, both doses of collagenase caused greater fatty deposits with respect to CTRL ($p<0.05$). Their increase was found at day 7 in the LD group and at day 15 in the HD group with respect to CTRL. The HD group showed higher levels of fatty deposits at all time-points, in particular, a significant difference was observed with respect to LD at day 15 ($p<0.05$). Overall, the LD-treated tendons showed a lower degenerative score with respect to the HD group. Moreover, the pathological changes in tendons treated with LD collagenase did not significantly vary during the experimental observation with any differences observed between time points neither for the total score nor for the specific features.

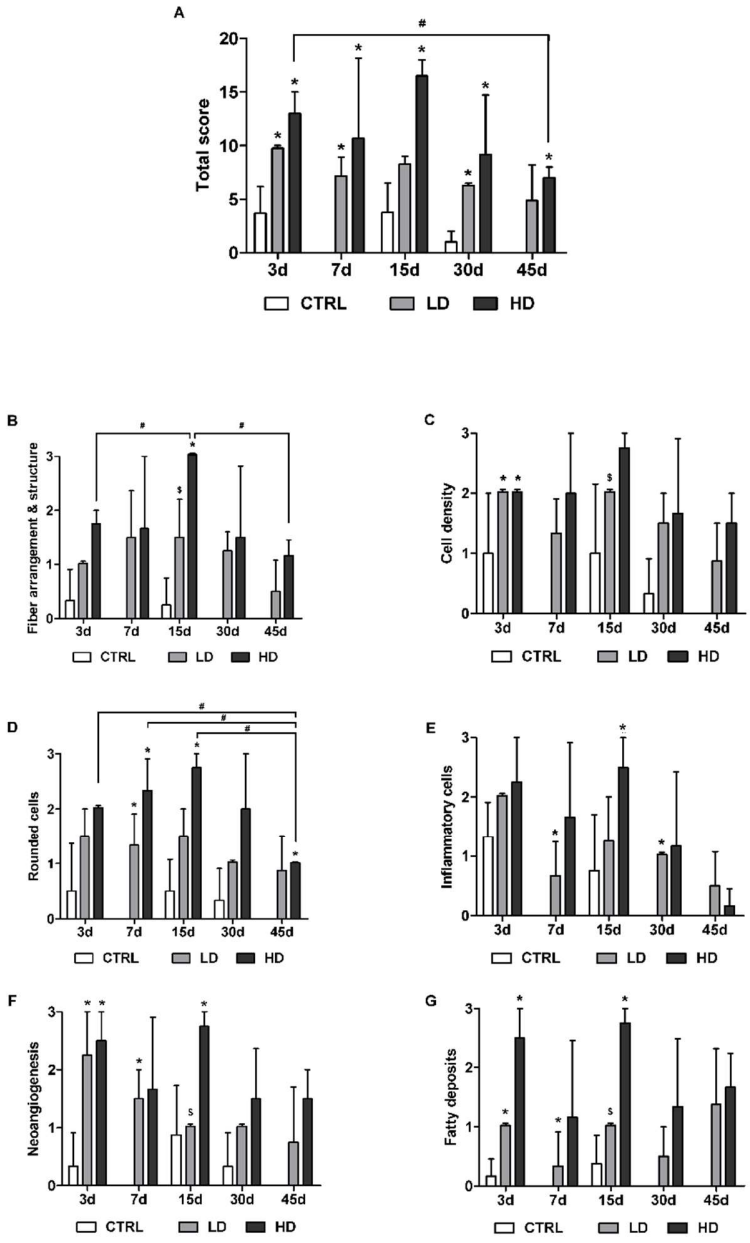

A
Total score
3d
7d
15d
30d
45d
CTRL
LD
HD
B
Fiber arrangement & structure
C
Cell density
D
Rounded cells
E
Inflammatory cells
F
Neoangiogenesis
G
Fatty deposits

Figure 4. *Histological scores. Total (A) and specific scores for fiber arrangement (B), cell density (C) and morphology (D), presence of inflammatory cells (E), neoangiogenesis (F) and fatty deposition (G). Data are reported as mean±SE. *p<0.05 respect CTRL; $p<0.05respect to HD; #p<0.05 respect to different time point; n= 4.*

DISCUSSION

Tendinopathy is an umbrella term that refers to tendon injury with unknown etiology [24]. More precisely, tendinopathy has been defined as tendinitis when a non-rupture tendon injury is associated with a very precocious inflammatory process [25]. This process brings to mechanical-related chronic lesions, commonly known as tendinosis [25]. The lack of knowledge about the physiopathology of tendinopathy leads to misleading opinions in the presence of a host inflammatory response and development phases of this disorder. In this context, the current failure to offer a complete clinical picture of such a multiple etiology disease [14, 26] increases the need of a valid animal model able to mimic the histological features of tendinopathy in humans and to establish a standardized tool suitable for future preclinical studies.

Collagenase is considered an effective method to induce the Achilles tendinopathy in preclinical models, as widely described in the literature [14, 15, 17-21, 27-30]. However, the use of different rodent strains, different protocols in terms of amount and type of collagenase administration and follow-up time points determined a poor reproducibility of the model [13].

Aiming at standardizing a rat model of collagenase-induced tendinopathy, we carefully compared the degenerative potential of two different concentrations

of collagenase type I. In particular, we provided a complete time course evaluation of collagenase-induced Achilles tendinopathy in rats, focusing on the tendon histopathology at different time points (3, 7, 15, 30 and 45 days), in order to define the most effective dose of collagenase at an exact temporal window able to generate histological evidences of tendon lesions.

The choice of the collagenase doses included in this work was based on our previous *in vitro* studies, in which the dose inducing the collagen fiber disruption was validated in tendon explants [31, 32].

Our results revealed that both LD- and HD-treated tendons displayed a disorganization of the collagen fibers and increased the number of rounded resident cells, suggesting that a single intra-tendinous injection was sufficient and effective to induce a prompt and severe impairment of Achilles tendon integrity, above all in tendons treated with HD of collagenase type I. Our findings were consistent with other studies performed in large animals, in which the severity of the pathology was related to the amount of the injected collagenase [33]. The morphological changes - especially occurring at day 15 - resembled the histological appearance of tendinopathy in humans. In fact, human Achilles tendinopathy exhibits disorganized and smaller collagen fibers, loss of their parallel orientation, and an increased amount of rounded-shaped tenocytes [6]. Overall, in this model, we demonstrated that the acute phase occurred from day 3 to day 15 and evolved towards a proliferative phase from day 15 to day 45 displaying a degenerative appearance associated to a very precocious and mild remodeling process, according to what observed by previous *in vivo* and human studies [6, 34]. Moreover, 25% of cases treated with both LD and HD collagenase showed the presence of chondrocyte-like cells disseminated within the damaged

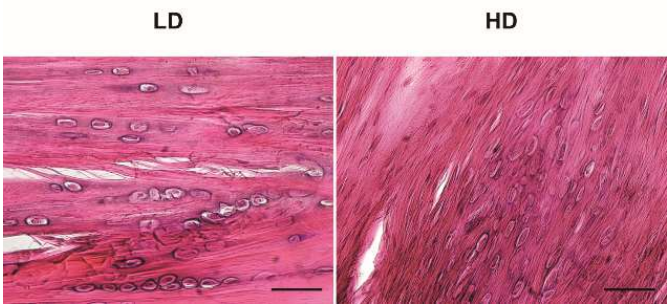
tendon fibers (see S1 Figure). This finding was consistent with other studies describing both the presence of chondrocytes and the up-regulation of chondrogenic genes in rat patellar and Achilles tendons after four weeks of collagenase injection [14, 27]. Similarly, chondrocyte markers were also expressed in human clinical samples of patellar [35], supraspinatus [36] and Achilles tendons [6]. The impairment of the tendon feature in favor of a fibrocartilaginous one assumes a pathological significance. Indded, the progressive lack of elasticity and tensile strength makes tendon more subjected to ruptures, even if no molecular mechanisms and pathways occurring in human Achilles tendinopathy have been investigated yet [37]. This condition was strengthened by some *in vitro* studies that demonstrated the capability of tendon stem/progenitor cells to effectively transdifferentiate towards the chondrogenic lineage [38-40].

The presence of fatty deposits was observed in tendons treated with HD collagenase, while they were rarely found in the LD-treated ones. The fat infiltration is retrieved in case of poor tendon repair. Moreover, fatty deposits were found in chronic tendinopathy in humans and in large animals [35, 41]. Our findings showed increased neo-angiogenesis in collagenase treated samples, more markedly visible in the HD-treated tendons than in the LD ones, above all at the earlier stages of the disease. The presence of new vessels was mainly restricted to the peritenon and it was combined with an initial increase of the cell number from day 3 to day 15, followed by a decrease from day 15 to day 45. The neovascularization and the increased amount of cells could be correlated to an inflammatory reaction. Our data were supported by several studies that demonstrated an inflammatory reaction in humans and animal models, both in

the early overload response and in the established tendinopathy [42]. However, the role of inflammation in tendon healing is still a greatly debated topic. Inflammation is highly beneficial to the tissue repair thanks to the release of cytokines and growth factors that together promote neoangiogenesis and the recruitment of resident and progenitor cells, and macrophages [43]. Nevertheless, how the inflammatory reaction can influence the progression of the pathology and how it can possibly contribute to the healing process are still unanswered questions. So, since its role in tendinopathy is uncertain, the presence (or absence) of an inflammatory response in our model would not have represented a crucial parameter to be considered in comparison with the human disease, thus, it was not deeply investigated. Despite our study was based only on histological evaluations, it was able to resemble the most important tendinopathy features in terms of tissue damages. These outcomes need to be examined in depth through quantitative analyses to assess the correlation of the histological findings with biochemical analyses, such as glycosaminoglycan, collagen and DNA contents, as well as with biomechanical parameters.

Overall, the results of this study suggested that the HD collagenase-induced tendinopathy is a reliable model in rats, resembling the human disease. In particular, our results demonstrated that collagenase type I efficiently induced three distinct stages of the disease over time, thus offering the opportunity to accurately investigate the pathological progression in a well-controlled establishment of this complex injury. More importantly, this model could be used to test novel therapies during the three-stage tendon disorder to achieve the most effective results in patients.

SUPPORTING INFORMATION



S1 Figure. *Chondrocyte-like cells in LD and HD treated tendon at day 45. Representative micrographs of the histopathological analysis; H&E staining. Scale bars 100 µm (20X).*

	0	1	2	3
Fiber structure and arrangement	Normal: continuous, parallel collagen fibers	Slightly abnormal: partially disorganized and fragmented fibers	Abnormal: moderately disorganized, fragmented, crossed and wavy fibers	Markedly abnormal: total disorganized and non-identifiable fiber pattern
Cell density	Normal	Slightly increased	Moderately increased	Markedly increased
Cell appearance	Spindle-shape cells	Slightly rounded cells	Moderately rounded cells	Markedly rounded cells
Inflammatory cell infiltration	<10%	10-20%	20-30%	>30%
Neovascularization	Normal presence of vascular bundles	Slight increase of vascular bundles	Moderate increase of vascular bundles	Marked increase of vascular bundles
Fatty deposits	Absence of lipid vacuoles	Slight increase of lipid vacuoles	Moderate increase of lipid vacuoles	Marked increase of lipid vacuoles

S1 Table. Grading system for the tendon histological evaluation

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5.2 Manuscript 9: Treatment of collagenase induced tendinopathy by Pulsed Electromagnetic Fields

Viganò M, Perucca Orfei C, Lovati AB, Bottagisio M, Di Giancamillo A, de Girolamo L.

This study is ongoing

INTRODUCTION

Tendon disorders represent a social and economic burden, for the high impact on worldwide population and at the same time the uncertainties in diagnosis and therapies [Hopkins C et al., 2016]. The need of new conservative treatments, beside pharmacological symptoms controls, recently led to the identification of biological effect of Pulsed Electromagnetic fields (PEMFs). The rationale to apply PEMFs in the treatment of tendinopathy is based on their effect on Tendon Stem/Progenitor Cells (TSPCs). In fact, PEMF induce proliferation of TSPCs and the production of anti-inflammatory cytokines (IL-10), pro-angiogenic (VEGF) and growth factors (TGFβ). Moreover, PEMF enhances the expression of tendon markers in the tendon cell population (SCX), also promoting a shift from the production of collagen type III to collagen type I [de Girolamo L et al., 2013; de Girolamo et al., 2014]. This substitution is crucial in the regenerative phase, when the scar tissue (collagen III) initially formed to fill the gaps in the extracellular matrix (ECM) is replaced by proper tendon tissue (collagen I) [Docheva D et al., 2015]. Taken together, these properties appear suitable to restore tendon tissue

homeostasis after a disruptive event. In this view, the working hypothesis of the present study is that experimental rat Achilles tendon lesion would heal more rapidly and that the newly formed tissue will present a better quality in rats exposed to PEMF with respect to untreated controls.

MATERIALS AND METHODS

In vivo procedures

The lesions were performed by collagenase type I injection (30 µl, 3 mg/ml in PBS) in the right Achilles tendon of 8 rats, as described before [Perucca Orfei C et al., 2016]. As control, in the left Achilles tendon of each rat, 30 µl of PBS were injected contextually. Rats were divided in 2 groups of 4 animals: PEMF and CTRL. Rats in the PEMF group were exposed to 8h of PEMF on daily base for 15 days, starting from 24 hours after the collagenase injection. The animals were sacrificed on day 16, as well as unexposed controls (CTRL).

Histological evaluations

The specimens were fixed in 10% formalin for 24h. Then, they were dehydrated in alcohol scale before embedding in paraffin and cutting into 5 µm longitudinal sections. Haematoxylin and eosin (H&E) staining was performed on all samples to evaluate the tendon morphology. An Olympus IX71 light microscope and an Olympus XC10 camera (Japan) were used to capture sample micrographs. Each sample was evaluated by one blinded observer to assess the tendon morphology according to the modified semi-quantitative grading score used in the previous article (Table 1) [Perucca Orfei C et al., 2016].

RESULTS

Histopathological findings

As expected, the use of collagenase induced a pronounced increase in fibers disorganization and cell density in both PEMF exposed and unexposed controls. Nevertheless, in unexposed tendons (Fig. 1A) the collagenase effect appear more evident with respect to PEMF treated ones (Fig. 1E). A closer look revealed also the presence of adipose degeneration (Fig. 1B) and neovascularization (Fig. 1F). The injection of PBS produced a mild effect on tendons, and indeed they appear less altered with respect to collagenase treated ones (Fig. 1C and 1G). At a microscopic analysis, no clear differences were identified between PEMF exposed and unexposed samples (Fig. 1D and 1H).

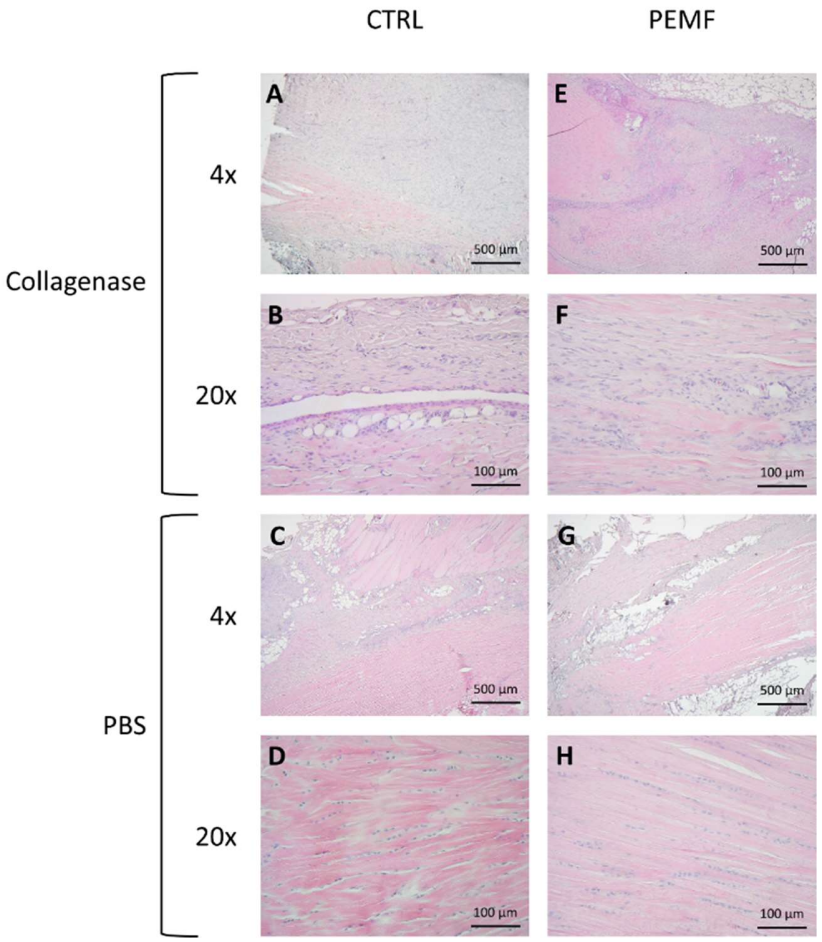

Figure 1. Histological appearance of tendons 15 days after collagenase type I (A,B,E,F) or PBS (C,D,G,H) injection. Representative micrographs of PEMF exposed (E,F,G,H) and unexposed (CTRL, A,B,C,D), at different magnifications.

Histopathological score analysis

The analysis of all samples in terms of histological score revealed an increasing tendency in collagenase treated samples compared to control PBS injected

tendons, in both PEMF and non-exposed groups (p=0.06 and p=0.08, respectively). Between the groups, a slight and non-significant decrease was observed for both PBS and collagenase treated tendons when they were exposed to PEMF, with respect to unexposed controls (Figure 2).

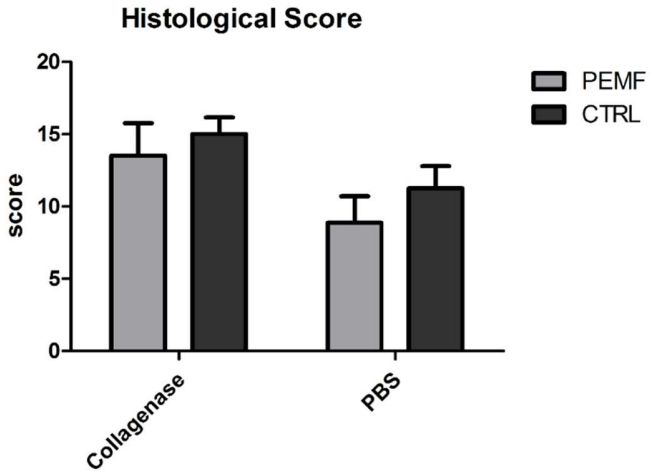

Figure 2. Mean histological score of tendons exposed to PEMF (PEMF) and unexposed controls (CTRL), treated with the injection of collagenase or PBS. Each group comprise the score of 4 tendons. Score range: 0-18, where 0 is native tissue.

Analyzing the single component of the histological score, the main differences were observed in the fiber arrangement, cell morphology and immune cell infiltration scores. For what concern fiber arrangement score, PEMF exposed tendons showed an improvement with respect to unexposed ones of -25% and -13% after collagenase or PBS injection, respectively. Cell morphology appear more physiological in PEMF group (-30 and -29%, after collagenase and PBS

injection, respectively) and the same was observed for the infiltration of immune cells (-25 and -33%).

DISCUSSION

The application of Pulsed Electromagnetic Fields represents a novel approach for the treatment of tendon disorders. The rationale resides in the pro-regenerative response that tendon cells showed after exposure to PEMF, which comprise the increase of cell proliferation, of the release of anti-inflammatory cytokines and trophic factors, and the enhancement in tendon specific markers expression [de Girolamo L et al., 2013; de Girolamo L et al., 2015]. To investigate whether the *in vitro* potential of PEMF would favor the *in vivo* tendon regeneration, a collagenase-induced tendinopathy was adopted as pathological model. This model is characterized by 3 phases, an acute phase (0-15 days after collagenase injection), a proliferative phase (15-30 days) and a regenerative phase (30-45 days). In this preliminary study, we decided to apply PEMF 24 hours after the lesion induction, in the acute phase, and maintain them 8 hours per day for 15 days. Despite the low number of animals treated, we were able to describe a slight improvement of histological appearance in PEMF-treated tendons, with respect to unexposed samples. In particular, the reduced infiltration of immune cells in PEMF group could be directly explained by the release of IL-10 observed *in vitro* [de Girolamo L et al., 2013], while TGFβ is possibly involved in the improved cell morphology due to its role in the expression of tenogenic markers [Pryce BA et al., 2009; Barsby T and Guest D, 2013]. The increase in collagen type I/type III ratio would explain the higher fiber organization observed in PEMF-treated tendons with respect to controls [de Girolamo L et al., 2015].

Despite the encouraging results obtained in this preliminary experiment, there are no sufficient data supporting the hypothesis of PEMF efficacy in the treatment of tendon lesions. Indeed, further analysis to increase sampling and assess the results at different time points are ongoing, as well as the investigation over the possible application of PEMF in different pathological phases. Moreover, the assessment of specific tenogenic and inflammation-related markers will improve the knowledge about the effect of PEMF in damaged tendons.

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6. Clinical application of MSCs from adipose tissue in the treatment of tendinopathy (*Aim4*)

Since the final aim of this research work is represented by the achievement of treatments that could be applied to patients, a clinical study was performed to evaluate the effect of MSCs-based treatment in tendinopathy. The clinical application of MSCs struggles among a series of regulatory issues, mainly regarding the difficult definition of these kinds of treatments in terms of pharmacovigilance, especially when cells are manipulated and thus are contextualized in the setting of advance therapy medicinal products (ATMPs). To avoid this, it is possible to exploit the potential as MSCs as freshly isolated progenitor concentrate from either bone marrow or adipose tissue. In this case the cells are just minimally manipulated and thus can be used with less limitations, although still in clinical trials and not in the consolidate clinical practice. In this research, freshly isolated stromal vascular fraction (SVF) from adipose tissue was administrated in a single-step treatment in the Achilles tendons of patients suffering from non-insertional tendinopathy refractory to other conservative treatments. The SVF represent a mixed population of cells

deriving from the digestion of adipose tissue, prior to the step of plastic adhesion selection, which comprises a variety of cells including adipose derived stem cells, pre-adipocytes, vascular smooth muscle cells, fibroblasts and endothelial cells. Since the use of collagenase for cell isolation is not allowed by European regulations for clinical application, the SVF was obtained by mechanical digestion of the tissue using a closed system commercially available. An *in vitro* evaluation of the anti-inflammatory ability of the SVF obtained by this method was coupled with the clinical observation to provide an explanation on the possible mechanism of action of MSCs in this pathological condition (Manuscript 10: de Girolamo et al., 2017, under review at KSSTA).

6.1 Manuscript 10: Intratendinous Adipose-derived Stromal Vascular Fraction (SVF) injection provides a safe, efficacious treatment for Achilles tendinopathy: results of a randomized controlled clinical trial at a 6-month follow-up

Usuelli FG, Grassi M, Maccario C, Viganò M, Lanfranchi L, Alfieri Montrasio U, de Girolamo L.

Knee Surg Sports Traumatol Arthrosc. 2017 Mar 1. doi: 10.1007/s00167-017-4479-9.

ABSTRACT

Purpose: Although platelet-rich plasma (PRP) injection has shown controversial results for the treatment of Achilles tendinopathy, it remains the most used biological treatment. Recent findings seem to demonstrate that the stromal vascular fraction (SVF) within adipose tissue may counteract the impaired tendon homeostasis. The aim of this study was to prospectively compare the efficacy of PRP and SVF injection for the treatment of non-insertional Achilles tendinopathy.

Methods: Fourty-four patients were recruited into the study; 23 of them were assigned to the PRP group whereas 21 to the SVF group, treated unilaterally or bilaterally for a total of 28 tendons per group. All patients (age 18-55 y/old) were clinically assessed pre-operatively and at 15, 30, 60, 120 and 180 days from

treatment, using the VAS pain scale, the VISA-A, the AOFAS Ankle-Hindfoot Score and the SF-36 form. The patients were also evaluated by ultrasound and magnetic resonance before treatment and after 4 (US only) and 6 months.

Results: Both treatments allowed for a significant improvement with respect to baseline. Comparing the two groups, VAS, AOFAS and VISA-A scored significantly better at 15 and 30 days in the SVF in comparison to PRP group ($p<0.05$). At the following time points the scores were not significantly different between the two groups. No correlation has been found between clinical and radiological findings.

Conclusions: Both PRP and SVF were safe, effective treatments for recalcitrant Achilles tendinopathy. The patients treated with SVF obtained faster results, thus suggesting that such a treatment should be taken into consideration for those patients who require an earlier return to daily activities or sport.

KEY words: Achilles tendon, Achilles Tendinopathy, PRP, Adipose-derived Mesenchymal Stem Cells, Stromal Vascular Fraction (SVF)

Level of evidence: Level 1 (Randomized Controlled Clinical Trial)

INTRODUCTION

The Achilles tendon is one of the most vulnerable tendons of the human body [22,37], often affected by tendinopathy, a multifactorial condition mainly related to overuse, degeneration and poor vascularization, representing 30–50% of all sports-related injuries [28]. In particular, non-insertional Achilles tendinopathies occur 2–6 cm proximal to the tendon insertion, which is an area characterized by a poorer vascularization. Modern research tools have contributed to creating convincing evidence that the inflammatory response is a key component of chronic tendinopathy. In particular, increased levels of macrophages, T- and B-

lymphocytes, macrophage-derived interleukin-1 (IL-1), cyclo-oxygenase (COX)-1, COX-2, IL-6, isoforms of transforming growth factor-β (TGF-β) and substance P were demonstrated in chronic Achilles tendinopathy [32]. Given the pathophysiological factors involved in chronic tendinopathy, the prospective use of biologic agents to enhance or restore healing in this condition has shown great promise. Platelet-rich plasma (PRP) is an agent that holds considerable theoretical appeal as a means of contrasting some of the mechanisms responsible for the development or persistence of the tendon lesions [12,25,38]. Many *in vitro* and pre-clinical studies have demonstrated that PRP may help stimulating angiogenesis, epithelialisation, cell differentiation, replication, proliferation and formation of extracellular matrix [3] since it contains several different growth factors (GFs), cytokines, chemokines and other proteins. Nevertheless, despite this positive and encouraging evidence, very conflicting results about the clinical effectiveness of PRP in tendinopathy are reported in literature, thus making it difficult to conclude whether PRP is an effective treatment [17]. However, the failure to demonstrate an evident therapeutic effect of PRP could be ascribed to an insufficient attention to patient selection, a lack of uniformity in term of tendinopathy severity and site of lesions and, above all, of univocal formulation of PRP [2,3,21]. Nevertheless, the scientific community still considers PRP as a potential useful tool to treat tendinopathy, as is demonstrated by the number of new ongoing investigations [40].

More recently, it has been demonstrated that many growth factors and molecules contained in PRP are produced and released by mesenchymal stem cells (MSCs) in response to a tissue injury or trauma. The recent discoveries indeed show that MSCs are associated to the perivascular district, they derive

from pericytes [6] and they secrete considerable levels of both immunomodulatory and trophic agents, besides having the ability to differentiate into different end-stage cell types [1,5,31]. In this view, MSCs can participate to tendon regeneration both by direct differentiation into tendon cells and, more likely, by modulating the inflammatory response following an injury [36,42]. Subcutaneous adipose tissue represents one of the most attractive sources to isolate MSCs due to a simple and less invasive method of harvesting, as well as the higher frequency of these cells within the stromal vascular fraction, if compared to bone marrow [9].

Pre-clinical studies have also demonstrated that adipose-derived stem cells (ASCs) provide significant improvements in the treatment of tendinopathy, suggesting the possibility to translate this approach to clinical applications [27,29].

The aim of this prospective randomized controlled trial was to compare the effectiveness of the injection of a leucocyte-rich PRP formulation with the injection of adipose-derived SVF for the treatment of chronic Achilles tendinopathy. The quality and duration of clinical improvement as well as of radiological findings were assessed at different time points up to 6 months after treatment. Moreover, the immunomodulation mediated by the SVF was tested in an *in vitro* model of inflammation.

MATERIALS AND METHODS

All patients provided a written informed consent and agreed to comply with a strict follow-up program. Patients of both sexes affected by non-insertional Achilles tendinopathy referring to the senior author's institution Foot and Ankle Unit were assessed for eligibility and prospectively enrolled in the clinical study.

The inclusion criteria were: unilateral or bilateral chronic tendinopathy of the Achilles tendon recalcitrant to traditional conservative treatments including non-steroidal anti-inflammatory drugs, eccentric loading exercises, stretching and biophysical therapy; symptoms lasting for at least 3 months; age between 18 and 55, VAS (visual analogue scale) pain at the first visit > 5. Patients with clinical suspect of other musculoskeletal lesions of the Achilles tendon (insertional disorders, tendon rupture or tears), platelet count in whole blood < $150X10^3/\mu l$, inflammatory disease or other conditions that affected the joints, immuno-mediated pathology, any conditions that could increase the interventional risk, use of tendon-detrimental drugs (i.e fluoroquinolones), patients who received any previous injective treatment of the target Achilles tendon, patients pregnant or breast-feeding were not enrolled in the study.

A week before receiving the treatment the patients were asked to suspend any non-steroidal anti-inflammatory drugs intake in order to prevent an impaired platelet function that may result in lower PRP quality regarding the content of bioactive compounds [35]. A day before the treatment, the patients who satisfied the inclusion/exclusion criteria of the protocol were randomly assigned either to PRP (n=28 tendons) or adipose tissue SVF (n=28 tendons) injection group, using opaque sealed envelopes previously prepared.

The patients were evaluated clinically pre-operatively and at 15, 30, 60, 120 and 180 days from treatment, using the 0-10 Visual Analog Scale (VAS) for pain (0 points, no pain; 10 points, worst possible pain), the Victorian Institute of Sport Assessment-Achilles (VISA-A) questionnaire,[33] the American Orthopaedic Foot and Ankle Society (AOFAS) Ankle-Hindfoot Score [16] and the Short Form (36) Health Survey (SF-36) forms. [4]

Preparation of PRP

PRP was prepared in the operating room using the GPS III System (Biomet Biology, Warsaw, IN, USA), a floating buoy-based separator system. Briefly, 54 ml of peripheral blood were collected from the patients enrolled in the PRP group and added to 6 ml of anticoagulant (ACD-A, 1:10 ratio). The whole blood was transferred to a disposable separation tube that was centrifuged at 3200 rpm for 15 minutes in a customized centrifuge provided by the manufacturer. Platelet poor plasma (PPP) was removed and platelets were suspended by gently shaking the tube for 30 seconds. The resulting PRP (around 6 ml) was extracted from the tube using a 10 ml syringe.

The platelet count in peripheral blood and PRP was analyzed for all the patients of the PRP group.

Preparation of adipose tissue stromal vascular fraction (SVF)

In the operating room a small volume of subcutaneous adipose tissue (50 ml) was manually lipoaspirated from the patient's abdominal subcutaneous tissue by an expert plastic surgeon, to ensure the quality of the harvest. Two very thin patients required to have adipose tissue harvested from the internal side of the thigh.

The SVF was obtained processing the adipose tissue with the FastKit system (Corios, San Giuliano Milanese, Italy), following the instructions provided by the manufacturer. The adipose tissue was transferred to a soft plastic bag with a 120 µm internal filter. Adipose tissue was mechanically digested rubbing the tissue down until it passed through the filter. The disrupted portion of the tissue, including the SVF, was collected through a bottom connector and then centrifuged for 10 min at 400 g. The resulting bottom phase (around 10 ml) was

then partially transferred to a new syringe of the volume required for the injection.

Platelet rich plasma and SVF injection

In the operating room, a volume of 4 ml of either PRP or SVF was injected into the lesion location, intratendon and in the peritendon area, by the senior author utilizing ultrasonography scanner with high frequency linear-array transducer (5.0-1.3 MHz), (Hitachi Hi Vision Preirus 14MHz, Hitachi Medical Sysytem, Milan, Italy). After the treatment, the patients were asked to walk with crutches for the first 24 hours and only paracetamol could be administered to control pain. No specific physical therapy was prescribed after the treatment and the patients were allowed to progressively resume their normal life and sport activities.

As per study protocol those patients who had presented a VAS > 3 and AOFAS < 70 at the 2-month follow-up visit were supposed to receive a second injection of the same product injected the first time.

MRI and US assessment

Patients were also evaluated by ultrasound (US) and magnetic resonance (MR) before the treatment and then after 120 days (US only) and 180 days. Both the radiologist and the clinical evaluator were blind to the allocation of the patients. Radiologic examination included US images and MRI scans of Achilles tendon. All radiological measurements were made using the standard tools provided by the Institute Picture Archiving and Communication System (PACS).

US images were performed pre-injection, and then at 120- and 180-day follow-up, with a 1.5 Tesla MR System (Avanto, Siemens Medical Solution, Milan, Italy). MRI scans of Achilles tendon were taken pre-injection and then at 6 months. In particular T1 (TR 400-750, TE 12-15) and T2 fat-saturated (TR 4000-5000, TE 70-

85) images in the sagittal planes were evaluated. The area of the lesion was analyzed and measured observing the maximum diameter as a reliable parameter for the lesion size (Figure 1-2). US and MRI were evaluated by a musculoskeletal radiologist and an orthopaedic foot and ankle specialist, both blind to the patient's treatment.

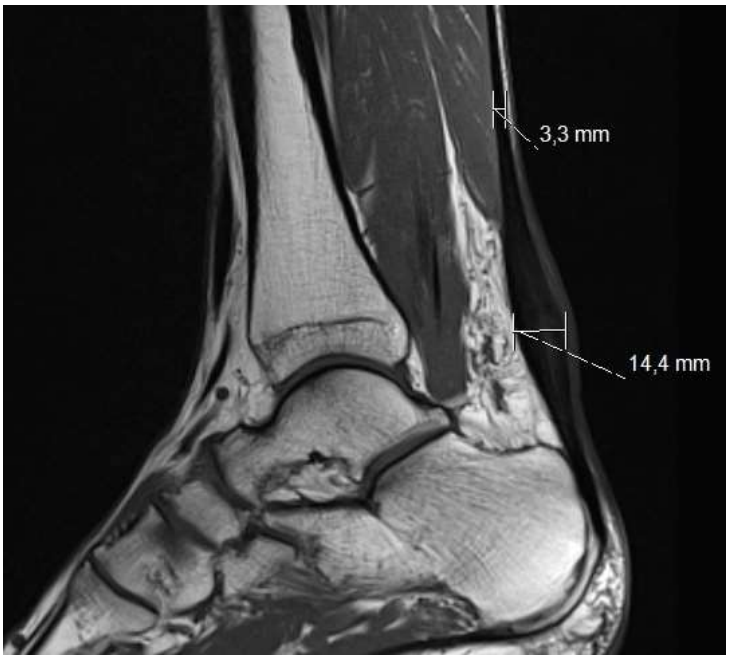

Figure 1: Pre-op MRI showing measurement locations in the sagittal plane.

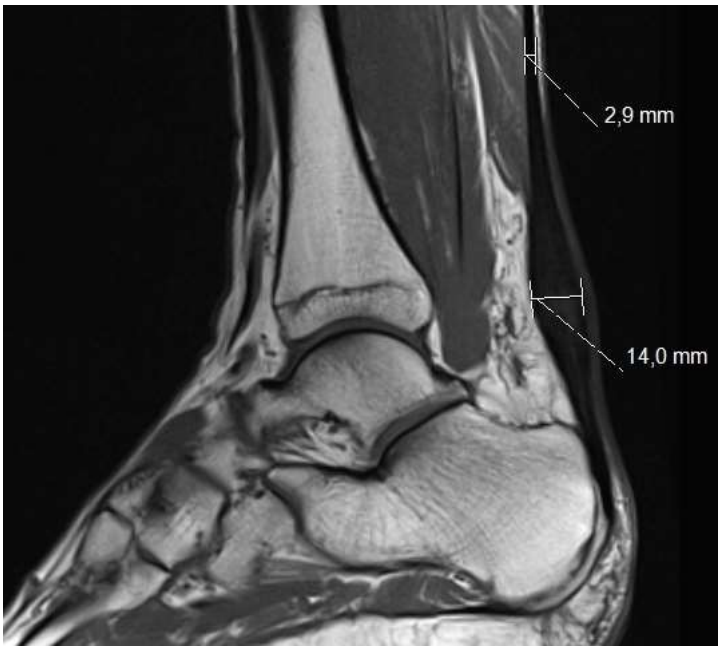

Figure 2: Post-op MRI showing measurement locations in the sagittal plane.

In vitro characterization of SVF immunodulatory potential

In vitro analysis on adipose tissue samples before and after processing by FastKit system were performed on 7 patients. Unprocessed adipose tissue was digested by collagenase type I to isolate adipose derived stem cells (ASCs) as previously reported [8]. Allogeneic peripheral blood leucocytes where isolated from 7 healthy donors not enrolled in the study under informed consent by Ficoll-isopaque technique (GE Healthcare, Little Chalfont UK). Briefly, 10 ml of peripheral blood was harvested from each donor, diluted 1:1 with sterile PBS and then centrifuged in falcon tube with 1:3 volume of Ficoll-isopaque. The

lymphocyte layer was then harvested, washed twice with PBS and then counted and seeded in RPMI culture medium (Sigma Aldrich, St Louis, MO, USA).

Allogeneic PBLs were cultured in the bottom portion of a 96-well transwell incubated at the density of 50,000 cells/well, in presence or absence of 10µg/ml Phytohemagglutinin (PHA; Sigma Aldrich), in order to stimulate the activation of the inflammatory response. A volume of 50µl of either the output from FastKit or from collagenase digestion was added in the top portion of the transwell. The same volume of processed tissue and re-suspension between FastKit and collagenase isolation protocols were maintained in order to make the comparison reliable.

After 24 hours of incubation, the production of IL-6 and IL-10, pro-infammatory and anti-inflammatory cytokines respectively, were analyzed in the supernatant by ELISA assay (Mabtech, Nacka Strand. Sweden). Limit of detection were respectively 10 pg/ml (intra-assay variation <4%) for IL-6 and 2 pg/ml (intra-assa variation <5%) for IL-10.

The study was approved by an external Ethics Committee (Azienda Sanitaria Locale, Milan- Italy; protocol number 24 bis-12 MS).

Statistical analysis

The normality of data distribution was tested with the Kolmogorov-Smirnov test. The sample size of the study was calculated considering a difference of 12 points and a standard deviation of 15 in the VISA-A score as a clinically significant difference between treatment groups [10,15]. Thus, accepting less than 5% probability of a type I error and a power of 80%, a total sample size of 25 tendons was required for each group. Predicting a 10% dropout rate, a total of 56 tendons were enrolled, equally divided in each group of treatment.

The statistical analysis was performed by use of Matlab statistical toolbox version 2008 (MathWorks, Natick, MA, USA) for 32-bit Windows. Differences between groups as well as within each group (preoperative vs postoperative in same group) were analyzed by use of Student t-test for unpaired and paired data, respectively. The Fisher exact test and a χ^2 test were used to compare categorical data. A p-value < 0.05 was considered statistically significant.

RESULTS

In vitro characterization of the immunomodulatory potential of SVF

The PBL expression of pro-inflammatory cytokine IL-6 was induced by PHA treatment. The addition of ASCs (from collagenase digestion) or SVF (from FastKit method) in the top portion of the transwell was able to significantly reduce the amount of IL-6 released in the supernatant by PBLs after 24 hours of incubation, by 36% ($p<0.01$) and 15% ($p<0.05$), respectively (Fig. 3a). An increase in IL-10 release was also observed in the supernatant of the samples treated with either ASCs (+128%) or SVF (+40%). However, these increases were not statistically significant due to the high interdonor variability (Fig. 3b).

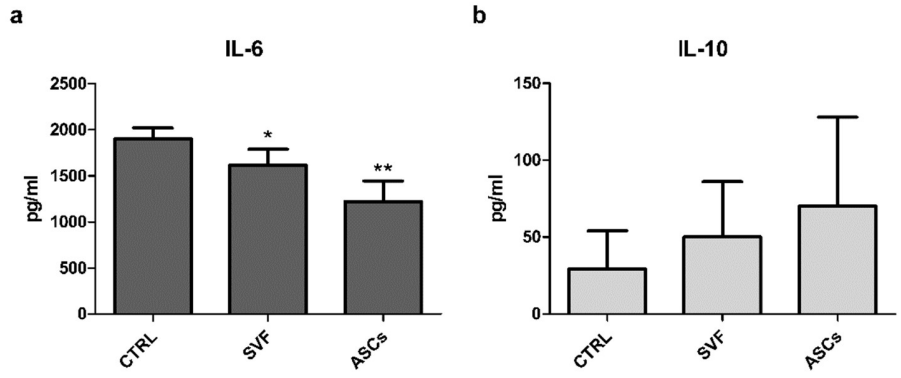

Figure 3: Production of IL-6 (a) and IL-10 (b) in a culture of PHA-activated PBLs, in

*presence or absence of ASCs (from collagenase digestion) and SVF (from Fastkit method) (n=7). *p<0.05 and **p<0.01 vs CTRL*

Clinical and functional results

Fourty-four patients were recruited into the study: 23 of them of them were assigned to the PRP group whereas 21 to the SVF group. A bilateral Achilles tendon injection was performed on 5 patients in the PRP group and in 7 patients in the SVF group, for a total of 28 tendons in each group (Fig. 4). All the patients were compliant with the study protocol with no loss during the study (Fig. 4).

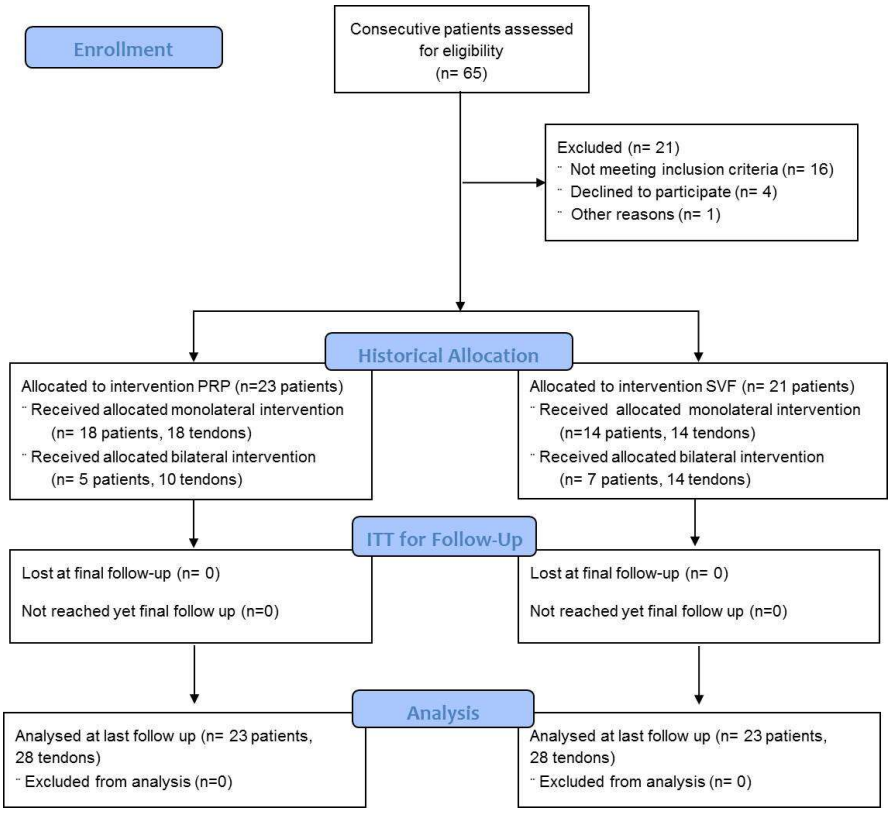

Figure 4: Consolidated Standards of Reporting Trials (CONSORT) flow chart for patient enrolment in the study

Both the clinical and functional background data were not significantly different between the two groups, with the exception of the sex ratio, with a higher prevalence of males in the SVF group with regards to PRP ($p<0.05$) (Table 1).

	PRP group	SVF group	*p*
VAS	6.3±1.2	6.5±1.6	*n.s*
VISA	46.5±23.6	41.6±13.6	*n.s*
AOFAS	63.2±17.7	63.4±20.1	*n.s*
SF-36 P	38.5±7.9	42.2±5.5	*n.s*
SF-36 M	51.21±8	48.7±5.7	*n.s*
Age	46.6±6.2	47.3±3.8	*<0.05*
Sex M - F	8 - 15	14 - 7	<0.05

Table 1: Background data of the PRP and SVF patients. Data are expressed as mean ± standard deviation.

Neither serious side effects nor adverse events were observed during the follow-up period. Five patients (25%) of the SVF groups also complained for hematoma and cutaneous discomfort at the adipose tissue harvest site for about a week after the procedure. At 2-month follow-up, all the patients had VAS and AOFAS score that met the study protocol requirement (> 3 and < 70, respectively), so no one received a second injection at the Achilles tendon.

The mean basal platelet count in peripheral blood of the patients included in the PRP group was $240\pm55X10^3/\mu l$; following the concentration procedure, the mean platelet count in PRP was $813\pm408X10^3/\mu l$, with an average fold increase of 4.14±1.9 with respect to the baseline.

During the follow-up, the patients of both groups had a significant improvement with respect to the pre-injection values. In particular, these improvements were faster in the SVF patients. Indeed, they showed significantly improvement for

VAS and AOFAS scale already after 15 days from the injection (Figure 5, 7) and VISA-A and SF-36 Score-Physical starting from 30 days (Figure 6, 8). In the PRP group the VAS scale scored better results already after 15 days, whereas significantly better results were observed only at a later time, in particular after 30 and 60 days for VISA-A and AOFAS, respectively. No significant improvement with respect to the pre-injection value was observed for the SF-36 Score–Mental in neither group (Figure 9).

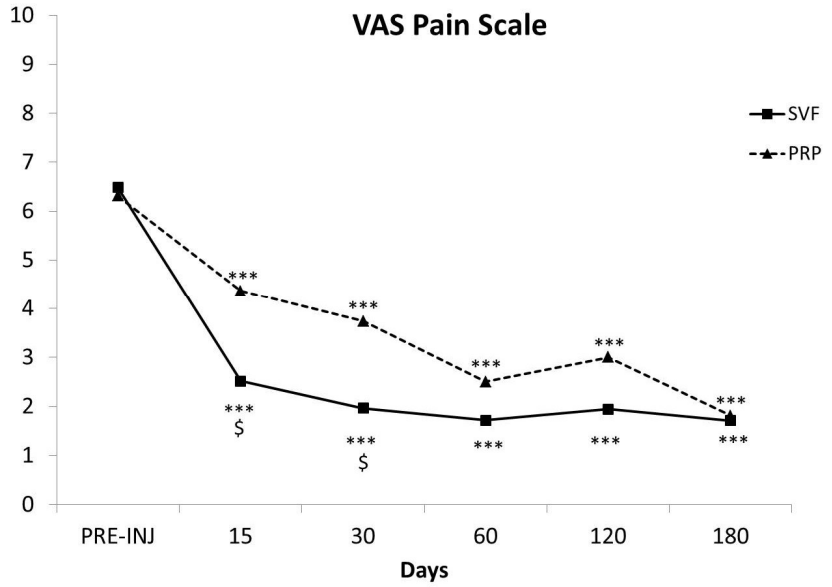

*Figure 5: Mean VAS score of patients treated with either PRP or SVF before injection and then during the follow up. ***, p<0.001 vs pre-injection; $, p<0.05 SVF vs PRP.*

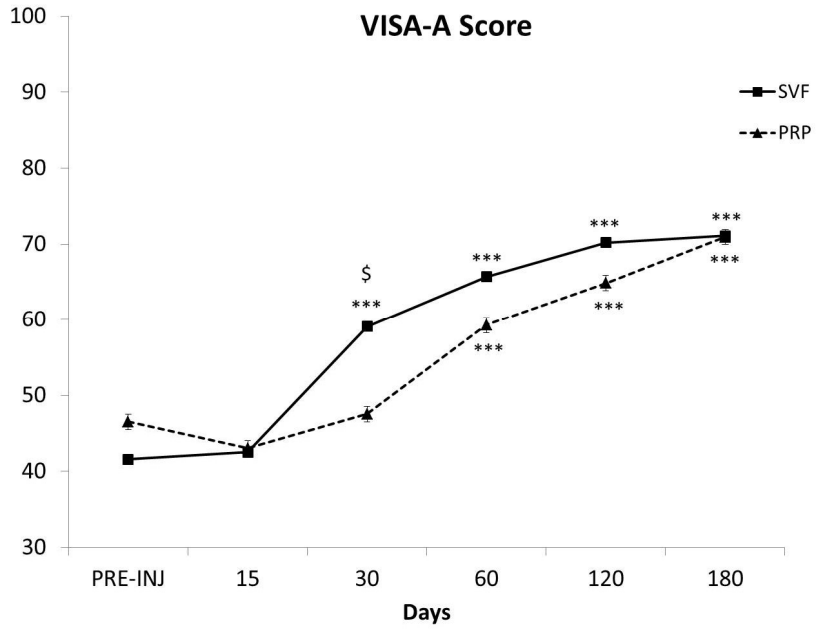

*Figure 6: Mean VISA-A score of patients treated with either PRP or SVF before injection and then during the follow up. ***, p<0.001 vs pre-injection; $, p<0.05 SVF vs PRP.*

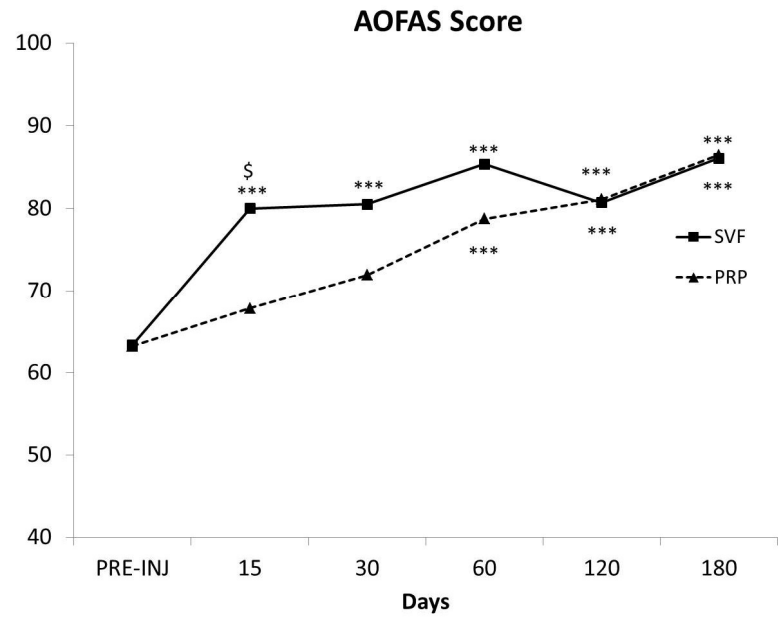

*Figure 7: Mean AOFAS score of patients treated with either PRP or SVF before injection and then during the follow up. ***, p<0.001 vs pre-injection; $, p<0.05 SVF vs PRP.*

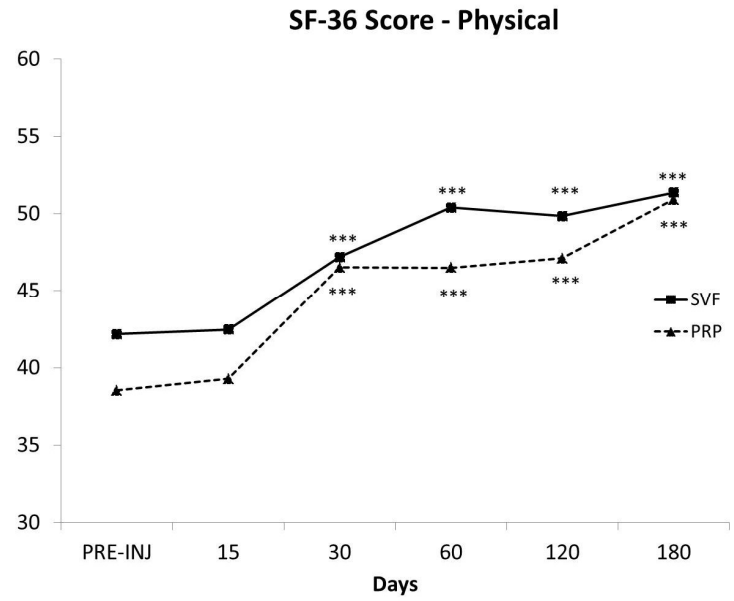

*Figure 8: Mean SF-36-Physical score of patients treated with either PRP or SVF before injection and then during the follow up. ***, p<0.001 vs pre-injection; $, p<0.05 SVF vs PRP.*

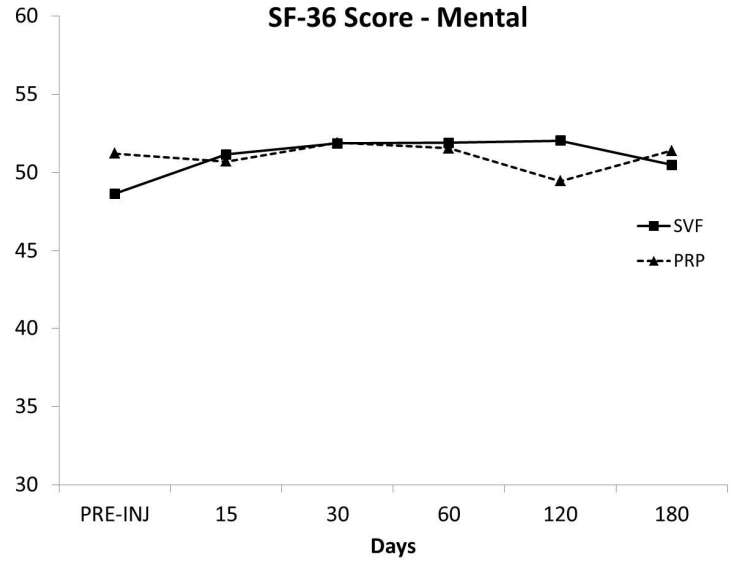

*Figure 9: Mean SF-36-Mental score of patients treated with either PRP or SVF before injection and then during the follow up. ***, p<0.001 vs pre-injection; $, p<0.05 SVF vs PRP.*

Consequently, a comparison of the two groups showed that statistically significant differences in favor of the SVF group were just found at the earliest follow-up: VAS scored significantly better at both 15 and 30 days in the SVF patients in comparison to PRP ($p<0.05$), as well as AOFAS and VISA-A at 15 and 30 days, respectively ($p<0.05$) (Figure 5-7). At the following time points the scores were not significantly different between the two groups anymore, even if SVF always scored slightly better than PRP.

US and MRI findings

Ultrasounds studies were a useful, reliable tool to identify tendon pathology and

lesion site pre- and peri-operatively. However, this diagnostic procedure did not allow for any relevant information regarding the lesion evolution during the follow-up.

The mean preoperative lesion area assessed by MRI were 8.93±2.10 mm^2 and 10.70±3.38 mm^2 in the PRP and SVF group, respectively (n.s). After 180 days from treatment, the lesion area was not significantly reduced in either PRP or SVF patients (8.67±2.10 mm^2 and 10.46±3.37 mm^2, respectively, n.s). These data demonstrated a lack of radiological improvement along the follow-up period, as well as any differences between groups at the final follow up.

No correlation between the pre- and post-injection lesion area and VAS, VISA-A, AOFAS, and SF-36 scores was found for both groups (n.s).

DISCUSSION

The main findings of this study showed that both the injection of a leucocyte-rich PRP and adipose-derived SVF have been able to provide a significant clinical improvement in term of pain relief and function restoration, with durable results for at least 6 months from treatment. Interestingly, the data yielded a faster recovery in the patients who had received the SVF injection, resulting in a significantly better outcome at 15- and 30 day-follow up in comparison to the patients of the PRP group. To our best knowledge, this is the first randomized clinical trial evaluating the effectiveness of adipose-derived SVF for the treatment of non-insertional Achilles tendinopathy. If indeed an increasing number of studies have demonstrated the potential of ASCs, either expanded or concentrated intraoperatively in form of SVF for the treatment of cartilage-related conditions [18, 23], very few data has been published to date in order to

assess the effect of these cells for tendinopathy.

The immediate pain relief already after 15 days from the treatment and the subsequent functional improvement observed in the patients treated with SVF seem to indicate that the treatment was able to counteract more quickly the impaired tissue homeostasis. These findings may be explained by a higher and longer-lasting activity of the anti-inflammatory and immunomodulatory molecules released by the cells within the SVF compared to the molecules contained in the PRP. However, at the latest follow-up the two groups showed no significant difference, although the SVF patients always scored better than the ones treated with PRP over the entire study period. Given the etiopathogenesis of tendinopathy, the wide anti-inflammatory and immunomodulatory properties of the molecules released by the cells within the SVF may provide alternative potential opportunities in treating chronic tendinopathies, replacing the traditional anti-inflammatory modalities (i.e NSAIDs)., MSCs secrete, indeed, a plethora of growth factors and anti-inflammatory proteins in response to inflammatory molecules, including prostaglandin 2, TGF-β1, hepatocyte growth factor (HGF), stromal cell-derived factor-1 (SDF-1), nitrous oxide (NO), indoleamine 2,3-dioxygenase, IL-4, IL-6, IL-10 and IL-1 receptor antagonist (IL-1 RA). [1,14,26,39,41] Moreover, MSCs prevent proliferation and function of many inflammatory immune cells, such as T cells, natural killer cells, B cells, monocytes, macrophages and dendritic cells [26]. Our *in vitro* findings confirmed these observations, showing a significant reduction in IL-6 production and an increase in IL-10 in an *in vitro* model of inflammation with peripheral blood leucocytes treated with SVF. The reason why the cells isolated with the traditional enzymatic method were able to further

induce this effect may be probably ascribed to the higher number of mesenchymal stem cells in the samples. Indeed, the FastKit method usually allows to obtain about ten times fewer cells than using the collagenase digestion method (unpublished data): this is consistent with the characteristics of the kit, being a faster but obviously less accurate method to obtain MSCs than the traditional laboratory technique. However, the clinical findings seem to indicate that the cells contained in the SVF were sufficient to promote a fast pain relief and a consequent restoration of the Achilles tendon function.

Although the use of a homogenous MSC population has been traditionally considered the best approach, today the maintenance of the architecture of the so-called stem cell niche seems to represent a great advantage [13]. The adipose SVF, the native microenvironment that contains pre-adipocytes, vascular endothelial cells, smooth muscle cells, leucocytes, erythrocytes and pericytes, including ASCs, in a collagen scaffold within a vascular network, is considered to be the ASC niche. In this microenvironment ASCs may be able to work in a more physiological condition rather than after being purified from the rest of the other cell populations within the tissue. Moreover, the use of the SVF over purified ASCs represent a practical advantage since the use of autologous expanded cells would imply a two-step procedure, characterized by higher invasiveness and cost, and would be subjected to more rigorous regulatory requirements for their use in clinical practice since it is considered an advanced-therapy medicinal product (ATMPs).

This study design aimed to compare the effect of a single SVF injection with another injective treatment as it was considered the most proper control group. Since needling is indeed an accepted form of therapy to induce healing in

tendinopathy of rotator cuff, Achilles tendon, patellar tendon as well as plantar fascia [19], if the SVF injection had been compared with a non-injective treatment, any positive effect derived from the SVF treatment might have been ascribed to the needling itself. Among the potentially effective injective alternatives, PRP was considered the best one since it has been used for about ten years for the treatment of tendinopathies, with a very high safety profile, even if conflicting results are reported in literature [24]. No side or adverse effects were indeed recorded during the study, neither in PRP, nor in SVF patients. Moreover, in many studies PRP has been reported to positively influence health-relevant outcomes, such as pain and disability, as also reported by a review showing that PRP injections ameliorated pain in the intermediate-long term compared with control interventions by pooling pain outcomes over time and across different tendons [2]. Still, these findings cannot be applied to the management of individual patients since they are affected by low power and precision [2]. In particular very good results have been obtained using a high-platelet concentration and leucocyte-rich product, especially for the treatment of epicondylitis [24]. For these reasons the same PRP formulation was chosen for this study, which is in any case one of the best studied [10,34]. Our results obtained in the PRP group confirmed the results obtained by other groups [7,11]. However, at the same time, other Authors showed a lack of significant difference between a PRP and a saline injection for the treatment of Achilles tendinopathy [10,20]. Such different outcomes can be ascribed to different inclusion criteria, evaluation methods, follow-up length, and poor number of patients as well as different study design. In a recent study, the Authors showed comparable results between an injection of PRP in Achilles tendons of 12 patients with respect to an

injection of saline solution in the same number of patients [20]. Even if the Authors initially meant to evaluate the outcome at a 12-month follow-up, they met with a considerable dropout and were just able to analyze the data after 3 months from the injection. Since our data showed that the functional and clinical results after PRP injections continued to increase during the evaluation period, reaching the best scores after 6 months, an earlier evaluation might have partially masked the beneficial effects of the PRP effects. Moreover, the huge discontinuation from that study could be ascribed to its design: indeed, the participants, who were blind to the treatment received, were told that they could leave the study and receive other treatments at any time after 3 months, if unsatisfied. In any case, in our opinion the lack of clarity concerning the effectiveness of PRP in Achilles tendinopathy deserve further investigations in hopes to finally reach a wider consensus.

Interestingly, in the present study no patient felt the need for a second injection. Although the patients included in this study were all affected by chronic tendinopathy, a single injective treatment, be it through PRP or SVF, indeed turned out to be very effective in relieving pain and quickly restoring functional properties.

Despite this clear positive clinical outcome, neither treatment group showed an improvement from a radiological point of view over the study duration, thus proving a complete lack of correlation between functional and radiological results. This finding is in line with most of the studies which failed to identify any morphological or histological change following the injection of biological agents in musculoskeletal disorders [23,30], whereas just few evidence seem to support a correlation between clinical and radiological improvements [28]. This may find

an explanation considering the strong anti-inflammatory and immunomodulatory properties of both treatments rather than a real regenerative action. However they could also be due to a lack in sensitivity of MRI and US in monitoring the healing process. In this study ultrasounds and MRI turned out to be useful instruments to support clinical diagnosis; further investigations are therefore needed in order to better analyze the real potential in the assessment of tissue regeneration.

One of the potential limitations of the study is the different sex distribution within the groups. Since the midportion Achilles tendinopathy equally affects women and men in the general population [9], this mismatch should however not have affected the results.

Since in many cases Achilles tendinopathy is bilateral, these patients were included in the trial too. In order to reduce the invasiveness of the treatment, both tendons were treated with the same products (PRP or SVF), so that patients just underwent either adipose or blood harvest. Another limitation could be represented by the lack of a control group treated with a saline injection. However, since the patients enrolled in this study were non-responders to the traditional conservative treatments and presented a moderate-high pain for some months (VAS >5), the treatment with saline was not considered ethical by the Ethical Committee.

Although a gap still exists between pre-clinical investigations and clinical applications, not only did these results show the safety, feasibility and rapid efficacy of biologic treatments in curing tendinopathy, they also particularly fostered the use of SVF for those patients who require to come back to high-demanding daily activities or sports earlier.

CONCLUSIONS: Both PRP and SVF were safe, effective treatments to cure recalcitrant non-insertional Achilles tendinopathy, with SVF allowing for faster results, already after 15 days from treatment. The lack of any correlation between clinical and radiological findings would deserve further investigations, including the research of more reliable evaluation tools of tendon healing as well as longer follow up to reach firm conclusions and extend the use of this biological product to the clinical practice

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7. CONCLUSIONS

The progression in MSCs research is testified by the growth of scientific publications, which increases on a yearly base since 1992, with the 66% of papers published in the last five years (https://www.bioinformant.com/two-thirds-of-all-mesenchymal-stem-cell-msc-publications-were-released-in-the-past-5-years/). The aspects that has fostered the great interest in this field include MSCs availability, therapeutic potential and versatility which make them a potential candidate to face different clinical needs. Most of these features, which contribute to the complexity of the investigations in this field, have been taken into consideration during my PhD path.

For what concerns MSCs availability, the description of stem/progenitors cells in human cartilage and tendon tissues represents an up-to-date contribution to the debate about cell-based therapy for tendon and cartilage repair. In particular, tissue specific progenitors might represent the most promising tool for Tissue Engineering of tendon and cartilage, possibly able to overcome the limitations showed by the use of adipose- and bone marrow- derived stem cells in these kinds of applications. Our findings demonstrated not only the presence of progenitor cells population in these tissues, but they also provided the proofs of concept

for their use as therapeutic agents and targets. Indeed, it appeared possible to increase the stemness, anti-inflammatory and tenogenic potential of Tendon Stem/Progenitor Cells with proper culture conditions and stimulations.

At the same time, years of research have demonstrated that the cells derived from human cartilage provide the most satisfactory results in cartilage regeneration [Oussedik S et al., 2015; Kon E et al., 2012]. Nevertheless, terminally differentiated chondrocytes possess a poorer secretory ability in comparison with ASCs. Thus, the use of MSCs from adipose tissue in particular, could provide the anti-inflammatory and trophic molecules that chondrocytes are not prone to produce. Moreover, our results show that ASCs could respond to an inflammatory environment with a catabolic response too. Indeed, MSCs can develop either anti- or pro- inflammatory phenotype depending on the kind of stimuli (or "priming") they are exposed to [Betencourt AM, 2013]. Therefore, the use of conditioned medium derived from properly "primed" ASCs emerges as a strong therapeutic opportunity to treat those pathologies characterized by chronic inflammation and degeneration, maintaining a high safety profile too. In this context, as demonstrated by our results cartilage tissue, and in particular articular chondrocytes or intervertebral disc cells, appear to be good targets for treatments based on MSCs conditioned medium.

Conclusions

The concept of "priming" of the MSCs comprises all the practices dedicated to tailor their action with the aim of enhancing their therapeutic properties. The condition of culture and selection, could determine great modifications in the behavior of MSCs and progenitors cells, such as premature senescence, phenotypic drift or, at the most extreme extent, the development of pro-inflammatory and pro-tumoral phenotypes [Betencourt AM, 2013; Bonab MM et al., 2006; De Veirman K et al., 2016]. For this reason, the control of culture conditions represents a crucial step, under both a safety and functional point of view, to apply MSCs/progenitor cells to the clinical practice. Since TSPCs are still a relatively underinvestigated cell type, the literature is still confusing about the proper protocols for their selection and culture. In our studies, we observed that the use of bFGF, which is often applied in culture of MSCs from different sources to increase their proliferation rate, lead to a decrease in the expression of stemness markers. Thus, its use should be avoided when cells are cultured for Cell Therapy purposes. Nevertheless, in different applications, this growth factor, alone or in combination with others such as BMP-12 and TGFβ3, resulted crucial in the induction of a tenogenic phenotype. Indeed, due to the lack of a consensus about the most effective tenogenic protocol, our results have had the value to improve the knowledge about the role of different molecules that would help the development of effective engineered tendon tissue for future clinical applications.

Other strategies to enhance the activity of MSCs towards a more efficient therapeutic behavior are represented by biophysical stimulations. Our results demonstrated that extracorporeal shock waves, known to induce bone healing by proliferation and osteo-differentiation of BMSCs and ASCs, are able to trigger both a paracrine response and a tissue specific marker expression in TSPCs [de Girolamo L et al., 2014]. Similarly, the exposure of TSPCs to PEMFs provided the same beneficial effects, with more pronounced effects in term of increase of collagen type I/type III ratio, a key point in the restoration of fully functional tissue after injury [de Girolamo L et al, 2015]. The great advantage of this approach is the possibility to enhance the MSCs *in situ* activity, overcoming a number of limitations related to invasiveness, cell culture methods and regulatory issues. This view would represent a third way, with respect to tissue engineering and cell therapy, considering MSCs as therapeutic targets. Indeed, while any tissue would retain the ability to self-regenerate to some extent, this is dependent on the number and activity of endogenous progenitor cells. Indeed, in a pathological environment or in the elderly, both number and features of progenitors could vary, reducing their tissue homeostatic capability [Lund TC et al., 2010; Peffers MJ et al., 2016; Ko KI et al., 2015]. In these conditions, the possibility to enhance MSCs number or activity by direct targeting with extracorporeal devices would have a great therapeutic significance.

To confirm the *in vitro* findings, we have developed an animal model of tendinopathy to test PEMF effects. Despite the large number of reports about collagenase-induced tendinopathy, before starting with the efficacy study we needed a complete characterization of pathological progression during time, which was not available so far. Our model allowed to identify different physio-pathological stages over the follow-up period, divided into an acute phase (0-15 days), a proliferative phase (15-30 days) and a regenerative phase (30-45 days). Starting from this evidences, we performed a small pilot study aimed to assess the efficacy of PEMF treatment (8 hours of exposure for 15 days) in a model of collagenases-induced rat Achilles tendinopathy administered during the acute phase. The study showed slight improvements in the histological appearance of PEMF-treated tendons with respect to unexposed controls. At the moment, we are evaluating the effect of PEMF when applied in different phases of the pathology, with the final goal to assess the most suitable timing for this kind of treatment. In the future, a deeper analysis will be performed to completely characterize the effect of PEMFs in this condition, comprising the assessment of specific molecular markers of each phase.

The final aim of any research in the applied biomedical science is the application of novel therapies to clinical practice. During my PhD research I had the great opportunity to participate in a randomized controlled clinical trial focusing on the use of cell therapy in patients suffering from

Achilles tendinopathy refractory to conservative treatments, providing my expertise to characterize *in vitro* the output of the intra-operative device used to process MSCs from adipose tissue (SVF). This analysis demonstrated that the adipose tissue progenitor concentrate was able to release immunomodulatory molecules. This result was confirmed clinically, as shown by the ability of SVF to promote a quick and durable improvement in term of pain and function in all the patients, with evidences of a superior effectiveness of this approach with respect to Platelet-Rich Plasma that was used as control.

Despite these promising results, the MSCs-based therapies remain a rapidly evolving field where open issues should be addressed with extreme awareness. Otherwise, the many hurdles between the MSCs research and their practical application could prevent them to keep the promises. My PhD research has contributed to prepare the background for the clinical translation of these scientific findings in the musculoskeletal field, addressing concrete problems regarding some aspects of MSCs-based approaches.

Further improvement of this work will include the combination of our tenogenic differentiation protocol with proper scaffolds to provide a complete tissue substitute to use in case of tendon tissue loss following Tissue Engineering approach. On the other hand, the application of different “priming” condition to Cell Therapy approaches will provide pathology-specific therapy to treat different diseases. In this context, the

direct use of cells will be probably replaced by the use of cell-free conditioned medium. Then, before achieving the ultimate goal of Regenerative Medicine, that is to provide novel clinically available therapies, the translatability of all these approaches will need to be proven in pre-clinical and clinical trials.

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